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PRINCIPAL INVESTIGATOR: Constantin G. Ioannides, Ph.D.

CONTRACTING ORGANIZATION: University of Texas
Houston, Texas 77030

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Houston, Texas 77030

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Studies during the last granting period have completed the analysis of identification of CTL epitopes in breast cancer. We have identified three antigenic systems: **(1.)** HER-2, **(2.)** folate binding protein, **(3.)** amino enhancer of split. These novel systems address the problem of the lack of antigenic targets for CTL in breast cancer. Furthermore, we analyzed the functional cytokine responses of CTL to these antigens. We found that stimulation of $CD8^+$ cells from either healthy donors or cancer patients with FBP or HER-2 CTL epitopes expressed either as peptides of as mini-genes in vaccinia virus vectors induces specific IFN-γ secretion. IFN-γ is an important mediation of anticancer effects. Ongoing studies focussed on enhancement of Ag presentation aim to overcome the difficulties in inducing complete activation and expansion of tumor Ag stimulated CTL, by focussing on post-translational modifications of the Ag.

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PROGRESS REPORT:

Introduction

Development of biological therapies for cancer in recent years has generated new hopes that improved cancer cure rates can be achieved beyond what is currently obtained with combination of chemotherapy and radiation therapy. Biological therapies use tumor Ag and cytokines (or their genes) with the objectives: **(1)** to induce tumor specific CTL and augment Ag presentation to anti-tumor effectors, **(2)** to achieve optimal proliferation and expansion of specific anti-tumor effector T cells (1), and **(3)** to ensure full activation of anti-tumor immunity.

Since CTL epitopes from tumor antigens are short peptides generated from self-proteins , to address the first objective, ongoing studies needed to accomplish general major tasks: **(1)** to characterize the tumor Ag either by mapping with synthetic peptides or by sequencing of the naturally processed peptides presented by the tumor **(1, 2)**, **(2)** to define approaches for presentation of these epitopes to tumor reactive CTL; **(3)** to identify the ability of tumor Ag presented in different forms (either as peptides, or genes encoding the peptides) to induce functional activation of responder $CD8^+$ cells in terms of cytolysis and cytokine production. **(4)** to enhance tumor Ag presentation.

The objectives of our studies during this grant period were five-fold **(1)** to complete epitope mapping in the AES tumor Ag system, by **(a)** defining the active epitope in the sequence identified by mass-spectrometry; **(b)** to develop cloned lines of $CD8^+$ CTL from breast TIL and characterize their recognition and fine specificity with regard to the AES CTL epitope; **(2)** to establish whether the Folate Binding Protein epitopes are immunogenic, capable of stimulating

the proliferation, cytokine secretion and cytotoxicity of breast and ovarian TIL; **(3)** to establish the requirements for endogenous loading of HER-2 peptides using mini-genes inserted in vaccinia vectors; **(4)** to characterize the tumor Ag presentation, by indentifying approaches to overcome deficiencies in Ag presentation.

The reasons for these investigations were five-fold: **(1)** the HER-2 antigen system although important covers only 30-50% of breast and ovarian cancer patients. Thus vaccines that will be developed based on HER-2 alone will benefit only a limited number of patients; **(2)** development of polyspecific vaccines which include complementary but distinct targets from HER-2 should benefit a larger segment of the patients population; **(3)** the proteins of the Notch (1-4) complex apparently are overexpressed/truncated in a large number of cancers. Thus the AES proteins may be similarly overexpressed because they are transcription factors, repressors of gene transcriptions. These repressors are activated by Notch engagement in a large number of cancers; **(4)** the folate binding protein (FBP) is overexpressed in > 90% of ovarian cancers and 50-70% of breast cancers. Thus a vaccine based on HER-2 + AES + FBP should be much more effective than a vaccine containing only HER-2; **(5)** since the wild-type and variant AES epitope differed by the N-terminal residue, it was important to know whether these specificities are overlapping or distinct; This is because for vaccine development purposes it is important to know whether we should use the variant protein (which is a limiting factor) only or the wild-type protein.

An important question deriving from tumor Ag identification is the ability of these Ag to sensitize $CD8^+$ CTL for activation of their effector function, i.e., establish that they are

functional in terms of cytolytic activity and cytokine secretion. These tasks are addressed in the FBP and HER-2 Ag systems. Two approaches were used: **(1)** presentation of the Ag in a peptide form and **(2)** presentation of the Ag in the mini-gene form. The mini-gene form used both the minimal epitope and the epitope fused with the adenovinus translocation sequences to facilitate transport through the endoplasmic reticulum. A major point of these studies was the investigation of the Ag presentation to T cells from peripheral blood mononuclear cells (PBMC) from healthy donors and breast cancer patients. The emphasis on healthy donors is because cancer vaccines should be more effective if used in breast cancer patients with no evidence of disease, in remission, or in healthy donors for the purpose of disease prevention. Thus these studies address, the questions of Ag presentation and functional activation of the effector CTL, by the Ag presented in different forms.

Furthermore, our studies on the effects of drug resistance on tumor cells, found that certain chemotherapeutic drugs such as Adriamycin may enhance the immunogenicity of some tumors. CTL immunotherapy may play a role in induction of reversion of drug resistance.

The results of these studies and their significance is presented in the Body of the report.

Body of the Report

(1.) The research performed during the last twelve months has made significant progress towards the goals of this study and overall goal of developing specific immunotherapy for breast cancer. The purpose of the present work continues to be the characterization of the functional significance of the CTL epitopes as potential antigens for targeted immunotherapy. The studies during this granting period focused on the well defined tasks.The results obtained are as follows:

1.1 Identification and Characterization of Tumor epitopes:

(a.) We have completed our studies on the characterization of the active epitopes of the Amino Enhancer of split (AES) protein of the Notch complex. The results of these studies have been submitted for publication. Their publications is pending in the Molecular Immunology (please see attached manuscript by Babcock et. al. and letter of acceptance).

The major topic of study during the past research period was to develop cloned lines from breast TIL and define recognition by these $CD8^+$ CTL of the wild-type (G75: LPLTPLPV) and variant (G76: GPLTPLPV) AES epitopes. The results shown in **Figure 1**, summarize the frequency and specificity of recognition of these clones indicating that breast TIL clones that specifically recognize the variant (G76) versus the wild-type epitope (G75) exist.

The results also show that the recognition by G76 specific CTL is Ag concentration dependent. These CTL-TAL recognize the epitope formed by G76 at concentrations as low as 0.1 μg/ml (~ 100 nM). These concentrations are significantly lower than the concentrations required to sensitize targets by most other peptides recognized by CTL in epithelial cancer **(3)** and the majority of the melanoma tumor Ag **(4).**

An important conclusion raised by our studies is the novel methodology for tumor Ag identification. Instead of growing increasingly higher number of tumor cells and adding additional HPLC separation cycles, our approach focus on defining the tentative sequence of the ions present, in an active peak, compare with overlapping synthetic peptides (which are faster and cheaper to obtain) and proceeds with functional assays. Thus we can provide a rapid answer to the question whether a candidate tumor Ag is recognized, i.e., is/was immunogenic in vivo.

(b.) We have completed our studies on the characterization of the active epitopes of the Folate Binding Protein (FBP). The results of these studies have been submitted for publication. Their publication is pending in the Annals of Surgical Oncology, and Proceedings of the National Academy of Sciences (please see attached manuscripts by Dr. Peoples and collaborators). Our results propose that since FBP is overexpressed 20-fold in most adenocarcinomas, these peptides may be utilized in a widely applicable peptide-based vaccine for epithelial tumors.

To evaluate the potential applications of FBP peptides for vaccine development – we investigated their ability to amplify the response of TAL. The FBP peptides E39 and E41 are efficient at amplifying the response of tumor-associated lymphocyte (TAL) populations in terms of lytic function, enhanced proliferation, and peptide-specific IFN-γ release **(Figure 2A, B)**. These studies addressed the question that the FBP epitopes are functional. Furthermore, on a per cell basis TAL stimulated with the FBP peptides exhibited enhanced cytotoxicity not only against peptide-loaded targets but also against FBP-expressing epithelial tumors of different histologies **(Figure 3)**. The broad distribution of FBP among >90% of ovarian and endometrial carcinomas, as well as 20-50% of breast, lung, colorectal, and renal cell carcinomas, along with pronounced differential expression in malignant tissues compared to the extremely limited expression in normal epithelium, suggests the exciting potential of a widely applicable FBP-based vaccine in epithelial cancers. (Please see attached paper, People et. al., 1998b).

(c.) Ongoing studies in active peptide characterization have identified the sequences of two other peptides mapped by ions of mass-to-change-ration: 1017 and 1008 **(5), (6)**. Because of the need to focus our studies on the functional aspects of the newly discovered epitopes, these studies will be continued in collaboration with Drs. Engelhard and Hunt (University of Virginia).

(2.) ***Endogenous and exogenous loading of HER-2 CTL epitopes.***

2.1: *Recombinant vaccina vector (rVV)*

The study of endogenous loading of HER-2 epitopes was performed in collaboration with Drs. Jonathan Yewdell and Jack Beninck from the laboratory of Viral Diseases of the

NIAID because of their expertise in the field (7, 8, 9). Since the results of this research are more recent, and have not been yet submitted for publication, a detailed presentation of these studies is made in this report. The sequences of the mini-genes encoding for the minimal determinant, E75, (designated as HER-2) and the minimal determinant containing the ER-translocation sequence ES-HER-2 as shown below.

ES-Her-2

Adenovirus E19 signal sequence

Sal I M R Y M I L G L L A
TCG ACC ACC ATG AGG TAC ATG ATT TTA GGC TTG CTC GCC
GG TGG TAC TCC ATG TAC TAA AAT CCG AAC GAG CGG

Her-2 antigenic determinant

L A A V C S A A K I F G S L A F
CTT GCG GCA GTC TGC AGC GCG GCC AAG ATA TTC GGA AGT CTA GCA TTC
GAA CGC CGT CAG ACG TCG CGC CGG TTC TAT AAG CCT TCA GAT CGT AAG

L stop stop *KpnI*
CTA TGA TA*G GTA CC*
GAT ACT AT*C CAT GGG TTC*
Sty I

Her-2

Her-2 antigenic determinant

Sal I M *K I F G S L A F L*
*TCG AC*C ACC ATG AAG ATA TTC GGA AGT CTA GCA TTC CTA
GG TGG TAC TTC TAT AAG CCT TCA GAT CGT AAG GAT

Stop stop *Kpn I*
TGA TA*G GTA CC*G C
ACT ATC CAT GG*C GCC GG*
Not I

In preliminary experiments we found that infection of T2 cells with the recombinant vaccinia virus (rVV) containing ES HER-2 failed to increase HLA-A2 expression. This was also true for the positive control peptide ESM1 (matrix M=58-66). Increase in HER-A2 expression was observed only when T2 cells were incubated after infection, with a proteasome inhibitor LLF. Proteasome inhibitors affect presentation of epitopes from proteins but not from short-peptides as demonstrated by Braciale and collaborators (10). The results in **(Table 1)** below, show that treatment of T2 cells infected with rVV, with LLF (11), lead to higher HLA-A2 expression in the case of rVV-HER-2 and rVV-ES-HER-2. A similar effect was observed for VV-ES-MI and for rVV-ICAM-1. rVV-ICAM-1 was used as positive control, for vaccinia virus driven antigen expression.

Table 1. Enhanced HLA-A2 stabilization after treatment of rVV infected T2 cells with LLF

	MCF			
Exp. 1	**HLA.A2**		**ICAM**	
	no LLF	LLF	no LLF	LLF
no rVV	406	122	77	79
rVV-HER-2	n	180	n	n
rVV-ES HER-2	276	197	n	n
rVV-ES MI	370	154	n	n
rVV-ICAM	318	134	131	238

Exp.2	HLA.A2		ICAM	
	no LLF	LLF	no LLF	LLF
no rVV	431	130	7.8	11
rVV-HER-2	366	143	nd	nd
rVV-ES HER-2	362	162	nd	nd
rVV-ES MI	377	146	nd	nd
rVV-ICAM	310	103	115	55

Targets were T2 cells infected with rVV at multiplicity of infection (MoI) ratio 400:1. Fluorescence analysis was performed 16 h later with HB54. Results indicate mean channel fluorescence MCF, for surface HLA-A2 or ICAM-1 expression determined by staining with mAB. LLF was used at 10 μm n, indicates not done. LLF (Z-carbo beuzoxy-L-leucyl-L-leucyl-L-phenylalanyl **(11)** is a proteasome inhibitor which preferentially inhibits chymotryptic activity. LLL **(12)** another proteasome inhibitor was used in parallel with less success (Data not shown).

To improve the yield of epitope presentation, (detectable by the stablilization assay), we attempted to inhibit viral DNA synthesis. We treated T2 cells with Psoralen (to inhibit DNA synthesis) **(13)** and extended the UV irradiation period of T2 cells infected with rVV-ES HER-2. Psoralen was used to generate non-replicating and noncytopathic rVV's. In the presence of longwave U.V. light, psoralen accomplishes this by targeting nucleic acid and introducing cross-links in the viral genome. This will allow the rVV to express small but not large genes.

Table 2. Treatment of rVV infected T2 cells with Psoralen failed to increase HLA-A2 and ICAM-1 expression.

	MCF						
	HLA.A2*						
	rVV-		**rVV-ESHER-2**			**rVV-ICAM**	
	-LLF	+LLF	-LLF		+LLF	-LLF	+LLF
CR19	112	112					
HER-2	111	110	15s	170	136	156	109
ES HER-2	107	97	30s	130	126	135	118
ES MI	98	103	60s	113	121	107	112
ICAM	106	nd	180s	105	122	111	117

* indicates that T2 cells were UV irradiated for 2 min.

CR19 indicates w.t. rVV without HER-2, matrix, or ICAM-1 mini-genes.

The results show that T2 treatment with Psoralen failed to increase HLA-A2 stabilization, even in the presence of LLF. Similarly, UV irradiation for longer time intervals failed to increase HLA-A2 expression. These results support a requirement for viral + cellular DNA synthesis for epitope presentation/plus a possible down regulative effect of HLA-A2 expression by the

vaccinia virus. Additional studies are ongoing to address the question of enhancing the epitope presentation plus reducing the rVV mediated toxicity.

These results demonstrated that tumor epitopes such as E75 can be presented after endogenous loading. However, the levels of presentation are significantly lower than when loaded exogenously as synthetic peptides.

To address the question whether T2 infected with rVV can induce IFN-γ secretion isolated $CD8^+$, from a healthy donor were used as responders (designated as healthy Donor 3, in the attached manuscript, Anderson et.al.). The results shown below **(Table 3)** indicate that peptide E75 pulsed T2 induced higher secretion of IFN-γ than T2 cells that were not pulsed with peptide (NP). None of the rVV constructs in the presence of psoralen induced higher levels of IFN-γ than NP, confirming the inhibitors effect of Psoralen.

Table 3 – IFN-γ induction from Donor 3 $CD8^+$ cells by rVV-E75

	IFN-γ(pg/ml/24hr)				
T2			DC		
+ Psoralen			- Psoralen	1	+Psoralen
NP	383				
E75	484				
CR19	156	washed	CR19	85.1	90
HER-2	144	washed	HER-2	284	81
ESHER-2	36	washed	ESHER-2	216	91
ESMI	109	diluted	CR19	0	ND
		diluted	HER-2	0	ND

Results in the first column were obtained with T2 cells as APC pulsed with peptide (first two values), or with T2 cells infected with control CR19, HER-2, and ESHER-2 rVV in the absence of proteasome inhibitors, but after two min UV irradiation and 10 μg/ml Psoralen. Results in the second and third column were obtained with DC as APC in the presence of LLF and Ara-C **(14)**. "Washed" indicate that DC were washed 6h after infection, to remove LLF, and Ara-C while "diluted" indicate that LLF, and Ara-C, were only diluted. T2 and DC treated with Psoralen were UV irradiated for 15 sec., 2 min. after infection.

The results show that when DC were not treated with Psoralen, and were washed after infection with rVV, higher levels of IFN-γ were secreted by healthy donor $CD8^+$ cells in response to HER-

2 than to ESHER-2. The HER-2 and ES-Her-2 mini-genes differ from CR19 (rVV) by the expression of the minimal determinant E75 or ER-E75.

These results support the hypothesis that IFN-γ is secreted in response to endogenously expressed E75. These results also show that: **(a)** IFN-γ secretion can be rapidly activated (within 16h) from $CD8^+$ cells of PBMC, suggesting that E75 primed activated memory cells exist in the healthy donors, and **(b)** the presence of proteasome inhibitors, psoralen, and (ara-c) in the IFN-γ induction assay inhibits IFN-γ secretion from $CD8^+$ cells. These results also indicate that activation of $CD8^+$ cells by endogenously expressed tumor Ag is feasible. However, a number of important technical details need to be addressed to optimize the immunogenicity of rVV containing mini-genes. Ongoing studies are attempting to address this point. The observation (point **(a)** above) is discussed in further detail in the following section (2.2. Exogenous loading).

2.2 *Exogenous peptide loading using antigen presenting cells (APC) or dendritic cells (DC).*

Most of this work has been completed and is presented in detail in the appended manuscript submitted for publication (Anderson et. al., submitted for publication). The major aspects of this work are summarized in this section.

Recent studies have shown that dendritic cells (DC) are the most efficient APC, for T cell activation, compared with macrophages or B cells. Therefore, we developed DC from plastic adherent PBMC by culture in GM-CSF and IL-4 for one week from healthy donors and breast

cancer patients and analyzed their ability to stimulate CTL functions in cytotoxicity and cytokine, (IL-2, IL-4 and IFN-γ) assays.

IFN-γ was rapidly induced in $CD8^+$ cells by DC-E75 in an E75 concentration dependent fashion, in the presence of IL-12, in all seven donors tested, even within 6 h of Ag exposure, but only in two donors in the absence of IL-12. Monoclonal antibodies to B7.1, B7.2 and CTLA-4 synergized with IL-12 in amplifying the IFN-γ response to E75 in all donors **(Figure 4 and 5)**. The major source of IFN-γ appeared to be *ex vivo* activated $CD8^+$ memory cells. Conversely, IL-12 and blocking of CTLA-4/B7.1 promoted only marginal proliferation of $CD8^+$ cells to E75, and did not induce/enhance antigen-specific lysis, neither in the donors with existent $CD8^+$ cells of E75 specificity (three of eight) nor in the five healthy individuals where such specificity was absent. The presence of functionally distinct, partially tolerized effectors, supports the existence of a diverse clonal composition dominated by different subpopulations in healthy individuals. Some of these cells may play an early role in protective immunity to the onset of IFN-γ sensitive HER-2 tumors. Similar results regarding functional activation of $CD8^+$ cells were obtained with PBMC from a breast cancer patient **(Figure 6, Appendix).**

These results taken together indicate that tumor Ag presentation either in the form of mini-gene or as synthetic peptide can activate the IFN-γ secretion function. However, these approaches, are not sufficient to stimulate tumor Ag specific CTL expansion and proliferation, when the responders are $CD8^+$ cells from peripheral blood of either healthy donors or cancer patients.

2.3 Identification of approaches to overcome deficiencies in Ag presentation.

Studies during this granting period have also focussed on the question of enhancing tumor Ag presentation. These studies were based on the hypothesis that since higher concentrations of antigen, and longer durations of Ag presentation are required to induce CTL than to sensitize CTL for lysis, modalities to increase the amount of Ag presented (detectable in CTL/cytokine assays) would enhance not only the tumor immunogenicity, but also provide a new approach for CTL stimulation. It should be noted from the results presented above, in the attached manuscripts, as well as from numerous recent studies that the poor induction of CTL expansion constitutes a major obstacle to successful cancer vaccination.

We focused first on the ability of Adriamycin, to enhance drug-resistance of tumor cells and their sensitivity to lysis by CTL. The results of this study are presented in the attached manuscript (Fisk et. al., Cancer Research, in press). These results show that, indeed, immunoselection of drug-resistant tumors with CTL, leaves the remaining tumor cells more sensitive to chemotherapeutic drugs. These novel findings also suggest a possible explanation for the results of earlier chemo-immunotherapy studies, where drug pre-treatment was expected to eliminate suppressor cells.

The increase in TAP expression, appeared not to be a major element in enhanced Ag presentation. While in breast and ovarian cancer the losses in TAP expression appear to

be less dramatic than in lung cancer, we observed that higher IFN-γ levels (which are known to increase TAP expression) do not enhance Ag immunogenicity.

This suggests that additional mechanisms are involved in epitope evasion. Our ongoing studies suggest that post-translational modification of the antigen (phosphorylation, ubiquination) direct the Ag degradation pathways. Ongoing studies using site-specific mutants of HER-2 with mutated phosphorylated sites are addressing this point.

Conclusions

The research during the previous period made the following contributors to the understanding of immunity in breast cancer and the completion of the proposal task (1-4).

(1.) Recognition by breast CTL-TIL, performed at clonal level has identified specific CTL lines and clones recognizing both the wild-type and variant AES protein.

(2.) Breast and ovarian CTL-TIL, were found to recognize Folate Binding Protein peptides. These peptides were stimulatory with regards to proliferation, cytolytic activity and cytokine secretion of $CD8^+$ CTL-TAL. Thus, we not only established the significance of HER-2 as a tumor antigen, but we identified two additional tumor Ag.

(3.) Mini-genes containing the minimal immunodominant HER-2 epitope, E75 have been developed. These mini-genes are currently being used to amplify tumor Ag immunogenicity. We found that enhanced presentation of the HER-2 epitope from mini-genes required proteasome inhibitors. Thus, it is possible that E75 competes with other epitopes for presentation by HLA-A2.

(4.) DC pulsed with E75 or infected with recombinant vaccinia virus containing the E75 sequence were able to activate $CD8^+$ cells from healthy donors to secrete IFN-γ. It should be mentioned that it is unlikely that the observed activation represents a primary response to Ag (peptide/virus). IFN-γ secretion was detectable within 16 h from the time

of stimulation. This indicate that a number of activated, HER-2 specific memory cells exist in healthy individuals (as well as in cancer patients). This is an important finding, and its impact is currently investigated both at the CTL and tumor level. Thus, we developed novel approaches for rapid functional activation of CTL by tumor antigen, which was the aim of the third task. We are currently exploring approaches to enhance the CTL expansion and proliferative ability.

(5.) Studies on enhancement of Ag presentation concluded that certain chemotherapeutic drugs such as Adriamycin enhance tumor sensitivity to CTL, suggesting that chemo and immuno therapy can synergize in certain instances in breast cancer patients. Furthermore, we found that E75 epitope presentation is negatively or positively regulated by phosphorylation of HER-2 at serine/theonine or tyrosine residues. Current studies are investigating the impact of HER-2 phosphorlation on immunogenicity.

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Appendices

List of Appended Publications:

1. Hudson, J.M., Castilleja, A., Murray, J.L., Honda, T., Kudelka, A., Singletary, A.E., Wharton, J.T., and Ioannides, C.G. Growth and antigen recognition by tumor infiltrating lymphocytes from human breast cancer. *Journal of Interferon and Cytokine Research*, 18:529-536, 1998.

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Abbreviations: OVTAL, ovarian tumor associated lymphocytes, BRTAL, breast tumor associated lymphocytes, AES, Amino enhancer of split; TLE, Transducin-like enhancer of split; PCR, Polymerase chain reaction; *m/z*, mass-to-charge ratio; U, mass unit; MS, mass spectrometry; w.t., wild-type; MCF, mean channel fluorescence. Folate binding protein, (FBP), cytotoxic T-lymphocytes, (CTL), tumor associated antigen (TAA), tumor associated lymphocytes (TAL), HER-2/neu, (HER-2)

Figure Legends

Figure 1. (A, B) Patterns of G76 and G75 recognition by $CD8^+$ CTL isolated by limited cloning from BRTAL-2. **(A)** $CD8^+$ CTL cultures preferentially recognizing peptide G75. **(B)** $CD8^+$ CTL cultures preferentially recognizing G76. Target T2 cells were pulsed with 1 μg/ml of G75 and G76 respectively. Because of the large number of cultures to be tested, for each culture, recognition of G75 (■) or G76 (●) was determined by an initial screening in the same experiment in duplicate. All resulting cultures were tested twice for recognition of G75 and G76. Only cultures which were confirmed in both experiments to preferentially recognize G75 or G76 were considered positive. The E:T ratio was 3:1. $CD8^+$ cells in the wells indicated as 5, 6, 7, 8 were initially seeded at 640, 320, 160 and 80 cells/well, respectively. Designations e.g., 27F indicate plate, column, row **(C)** Concentration- dependent recognition of G76 by line B27E isolated from a $CD8^+$ culture previously found in a separate experiment **(B)** to preferentially recognize G76 over G75. The E:T ratio was 2:1. (■) G75, (●) G76; **(C)** Recognition of G76 was significant compared with that of G75 at both 0.1 and 1.0 μg/ml ($p<0.05$). The results show the means and standard deviations of one CTL assay performed in triplicate.

Figure 2A. Stimulation with FBP peptides results in enhanced OvTAL proliferation and IFN-γ release. (A) OvTAL1 and OvTAL2 respond differently to FBP peptides. E39 (▦) and E41 (▮). OvTAL were cultured in parallel, and stimulated with irradiated T2 loaded with E37 (□), E39 ( ), E41, or no peptide (NP) (□). Cell counts were performed after one week. Results are expressed as proliferation index, calculated by individually comparing the E39 and E41-stimulated cultures to the NP-stimulated parallel controls. (B) OvTAL1 and OvTAL2 demonstrate peptide-specific IFN-γ release. Parallel cultures of OvTAL-1 or OvTAL2

stimulated with T2 loaded with E39, E41, and NP were replated at 1 x 10^6 cells/ml in 1 ml and restimulated with T2 and the corresponding peptides E39, E41, E37 or T2/NP IFN-γ and IL-4 were measured 24 and 48 hours later. Results shown for IFN-γ are expressed as pg IFN-γ /ml as measured by ELISA from 50 μl culture supernatant. This result is representative of replicated experiments.

Figure 2B. Enhanced cytotoxicity after FBP peptide stimulation. (A) Three OvTAL populations were stimulated in parallel with irradiated T2 loaded with E39 ( ) or T2/NP ( ). Recognition of E39 was assessed in standard CTL assays after one week at an E:T of 10:1. Results are expressed as % specific lysis ± SEM. (B) OvTAL2 was split, cultured in parallel, and stimulated with irradiated T2 loaded with E37 ( ), E39 ( ), or E41 ( ). After one week, these cultures were tested at an E:T=10:1 for recognition of these same peptide-loaded T2 targets in 5 hr CTL assays and of the tumor target, SKOv3.A2, in 20 hr assays. Results are expressed as % specific lysis ± SEM. ND=not done. (C) OvTAL1 was similarly stimulated as OvTAL2 in parallel with irradiated T2 loaded with E37, E39, E41, or NP twice at weekly intervals. The resulting cultures were tested in standard 5 h (C) and 20 h (D) cytotoxicity assays for recognition of peptide-loaded T2 and SKOv3.A2 at a lower E:T ratio of 2.5:1. Results are expressed as % specific lysis ± SEM. ND=not done.

Figure 3. FBP peptide stimulated ovarian TAL recognize FBP-expressing tumors of different histologies. (A) OvTAL2 stimulated twice at weekly intervals with irradiated T2 loaded with E37, E39, or E41 were tested for recognition of the low HLA-A2-expressing breast cancer line SKBr3.A2 (HLA-2^{low}, HER2/neu^{high}, FBP^{+}), ( ) the ovarian cancer line SKOv3.A2 (HLA-

$A2^{high}$, $HER2/neu^{high}$, FBP^{+}), ( ) and the colon cancer line SW480 ($HLA\text{-}A2^{high}$, $HER2/neu^{low}$, FBP^{+}) ( ) in standard 20 hr cytotoxicity assays at an E:T ratio of 2.5:1. Results are expressed as % specific lysis $\pm$ SEM. (B) OvTAL1 was stimulated twice at weekly intervals with irradiated T2 loaded with E39 and tested for recognition for the same tumors above and also the pancreatic cancer cell line, PAN-1 ($HLA\text{-}A2^{high}$, $HER2/neu^{high}$, FBP^{+}) in standard 20 hr cytotoxicity assays at an E:T ratio of 2.5:1. Results are expressed as % specific lysis $\pm$ SEM.

Figure 4. Freshly isolated plastic non-adherent PBMC form Donor 2 specifically secrete IFN-γ in reponse to stimulation with E75. Potentiation by IL-12, anti B7.1 and B7.2 mAb. The HLA-A2 restricted HER-2 peptide E75, and the control peptides E39, D132 and F57 were used at a final concentration of 10 μM. IL-12 was used at 3 U (330 pg/ml). IL-2 was used in the experiments shown in parallel A and B at a final concentration of 5 Cetus units (30 IU/ml) for the last 8 h of culture before collection. Results in A, B and C are from separated experiments.

Figure 5. Early and rapid induction of IFN-γ in plastic non-adherent PBMC from Donor 3 is IL-12 and peptide concentration dependent. Results in panels A and B are from the same experiment. (A, B) All peptides were used at 10 μg/ml; IL-12 at 330 pg/ml (A-D). IL-12 and anti CTLA-4 mAb synergize in enhancing IFN-γ induction in response to E75 in peptide isolated $CD8^{+}$ $CD45RO^{+}$ cells. Equal numbers of $CD8^{+}$ $CD45RO^{+}$ and $CD8^{+}$ CD45RO cells

were used as responders in each well. Experimental conditions were as described in the Materials and Methods.

Figure 6. Rapid induction of IFN-γ in $CD8^+$ cells of a breast cancer patient. APC were autologous DC from the PBMC of this patient. Experimental conditions as described for healthy Donor 2 and 3. Please note the synergistic effects of IL-12 and anti B7-1 mAb in inducing rapid IFN-γ secretion.

FIGURE 1

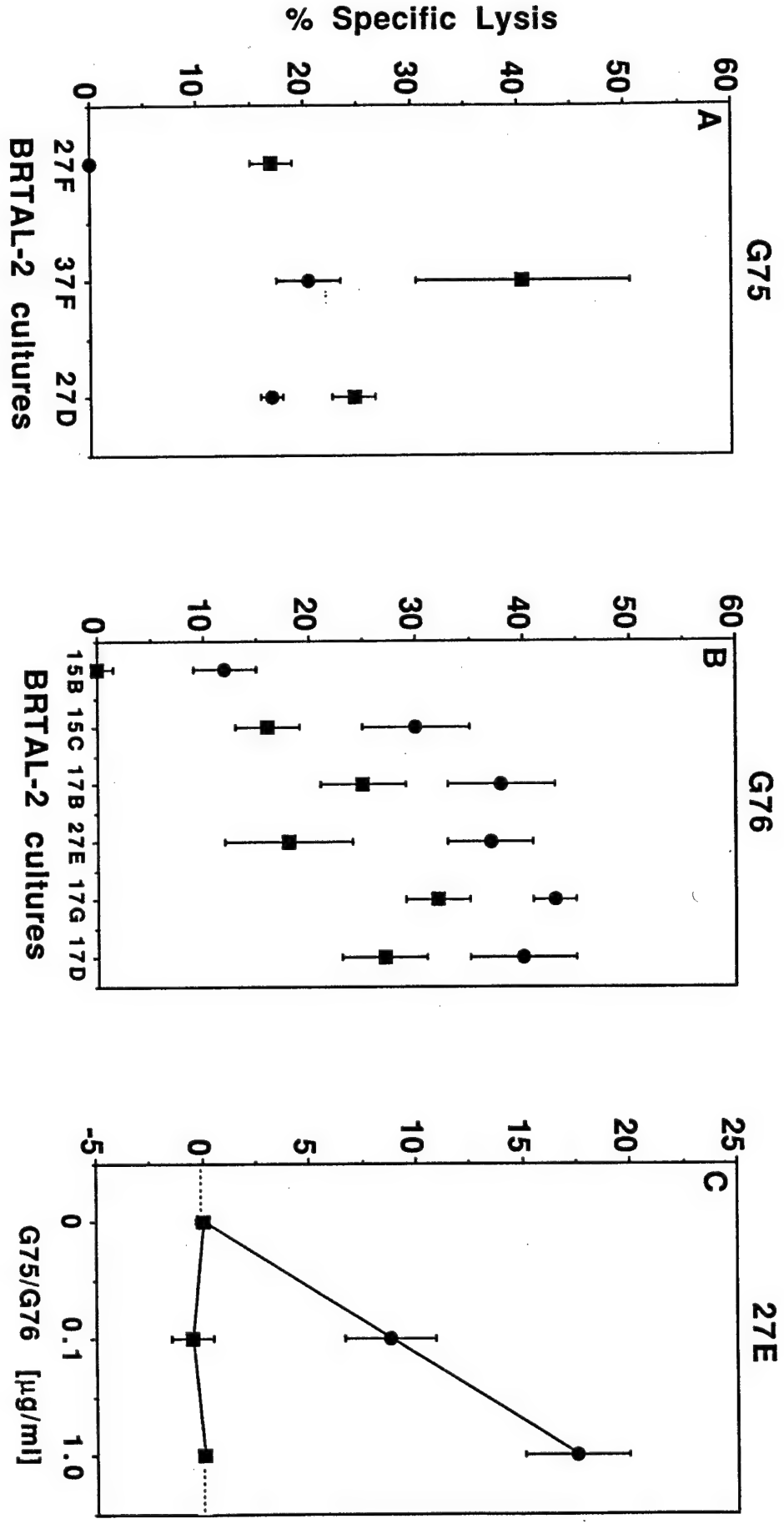
% Specific Lysis
A
G75
BRTAL-2 cultures
27F
37F
27D
B
G76
BRTAL-2 cultures
15B
15C
17B
27E
17G
17D
C
27E
G75/G76 [µg/ml]

FIGURE 2A

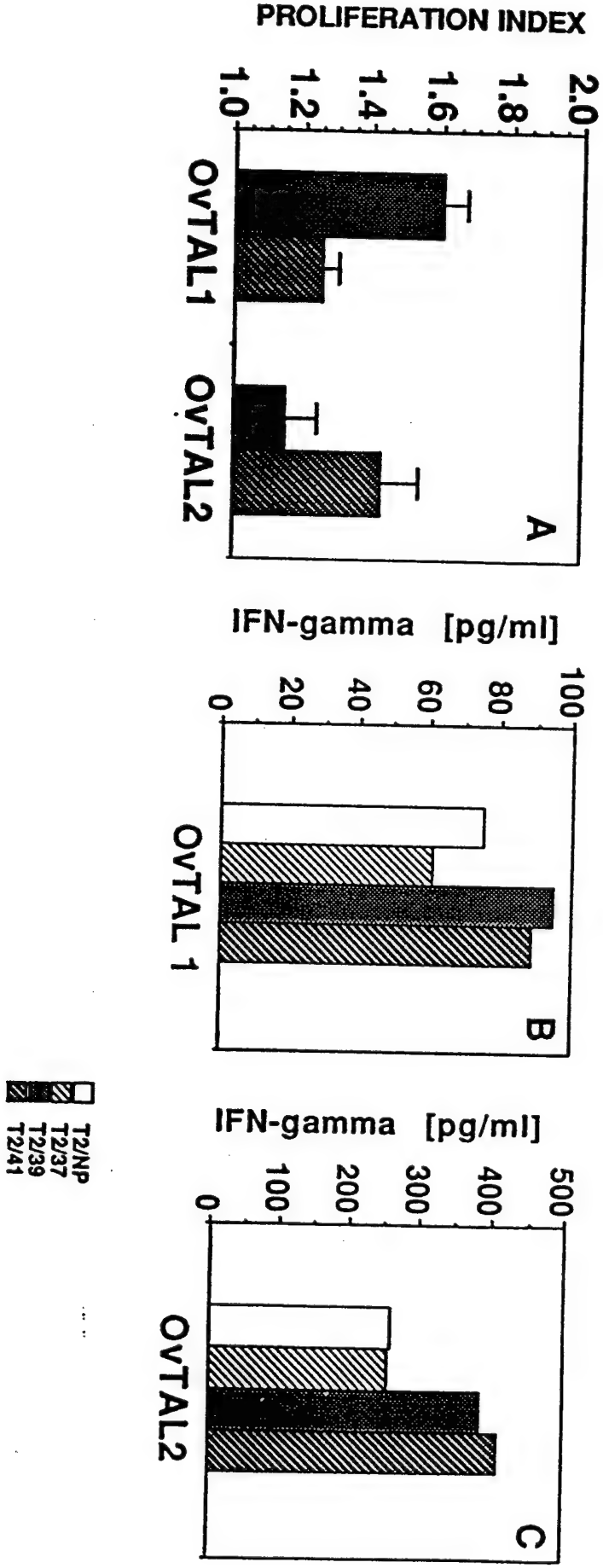
PROLIFERATION INDEX
1.0
1.2
1.4
1.6
1.8
2.0
OvTAL1
OvTAL2
A
IFN-gamma [pg/ml]
0
20
40
60
80
100
OvTAL 1
B
T2/NP
T2/37
T2/39
T2/41
IFN-gamma [pg/ml]
0
100
200
300
400
500
OvTAL2
C

FIGURE 2B

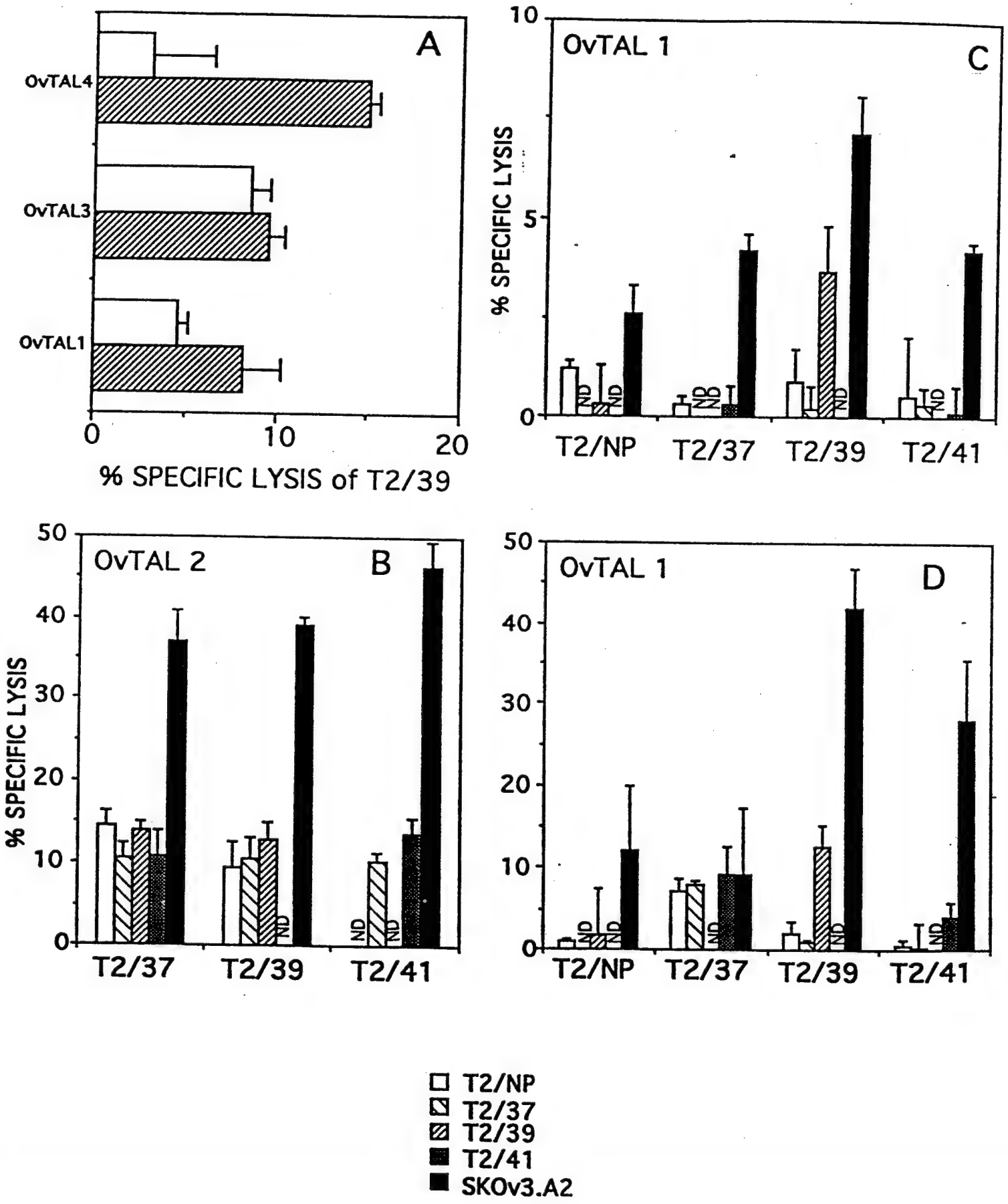
A
OvTAL4
OvTAL3
OvTAL1
0
10
20
% SPECIFIC LYSIS of T2/39
OvTAL 1
C
% SPECIFIC LYSIS
10
5
0
ND
T2/NP
T2/37
T2/39
T2/41
OvTAL 2
B
50
40
30
20
10
0
% SPECIFIC LYSIS
ND
T2/37
T2/39
T2/41
OvTAL 1
D
50
40
30
20
10
0
ND
T2/NP
T2/37
T2/39
T2/41
T2/NP
T2/37
T2/39
T2/41
SKOv3.A2

FIGURE 3

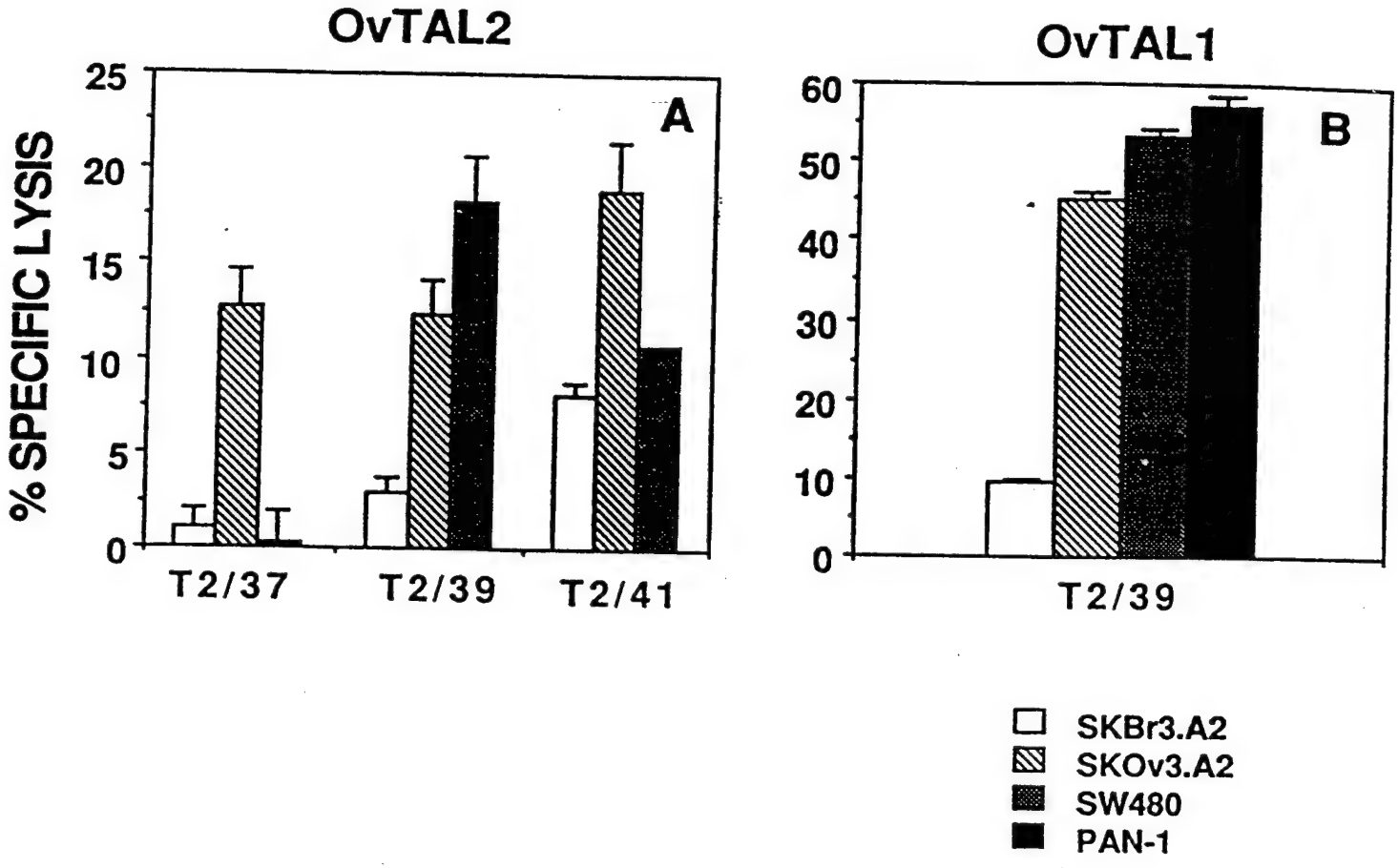
OvTAL2
% SPECIFIC LYSIS
25
20
15
10
5
0
A
T2/37
T2/39
T2/41
OvTAL1
60
50
40
30
20
10
0
B
T2/39
SKBr3.A2
SKOv3.A2
SW480
PAN-1

FIGURE 4

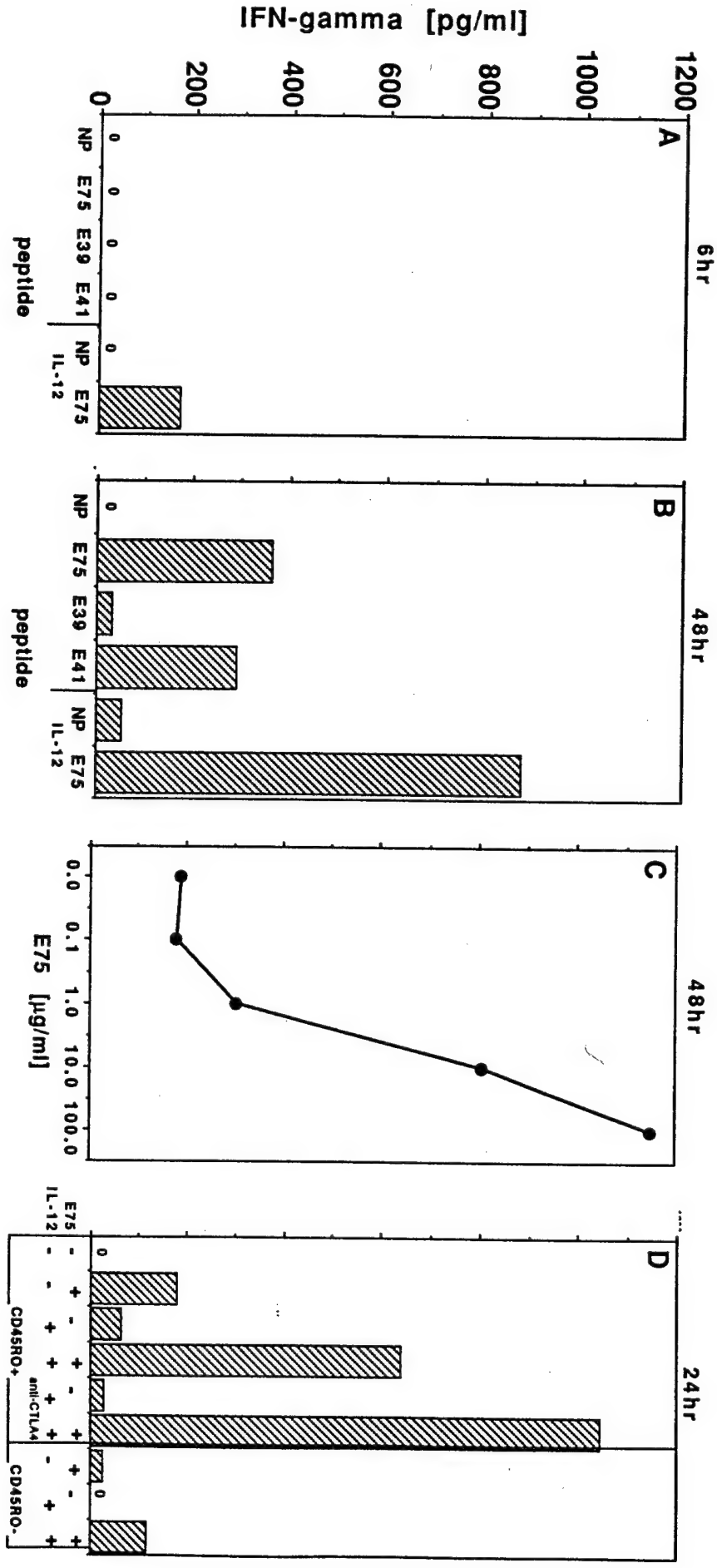
IFN-gamma [pg/ml]
1200
1000
800
600
400
200
0
A
6hr
NP
E75
E39
E41
NP
E75
peptide
IL-12
B
48hr
C
48hr
0.0
0.1
1.0
10.0
100.0
E75 [µg/ml]
D
24hr
E75
IL-12
anti-CTLA4
CD45RO+
CD45RO-

FIGURE 5

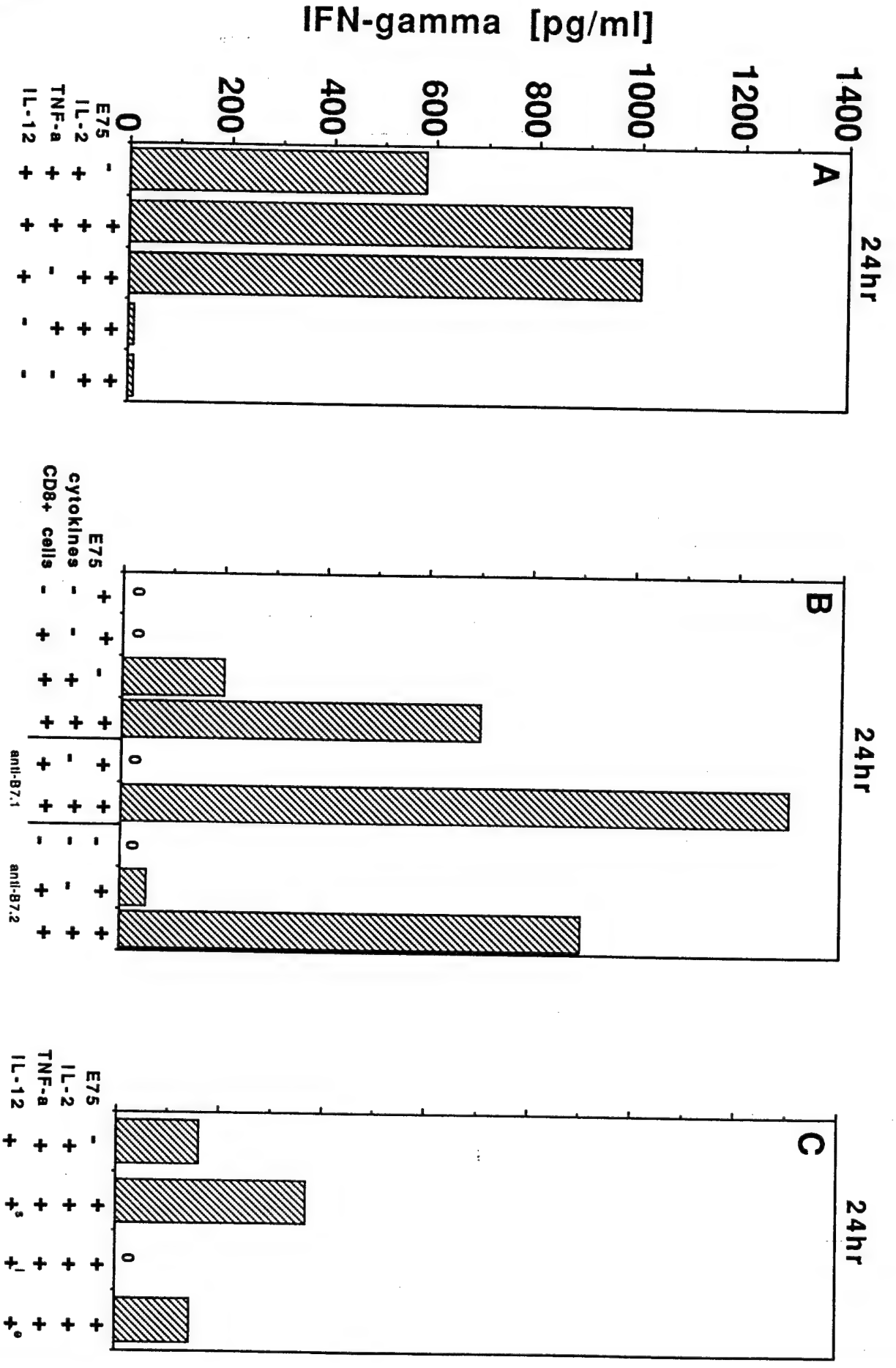
IFN-gamma [pg/ml]
1400
1200
1000
800
600
400
200
0
A
24hr
E75
IL-2
TNF-a
IL-12
B
24hr
E75 cytokines
CD8+ cells
anti-B7.1
anti-B7.2
C
24hr
E75
IL-2
TNF-a
IL-12

FIGURE 6

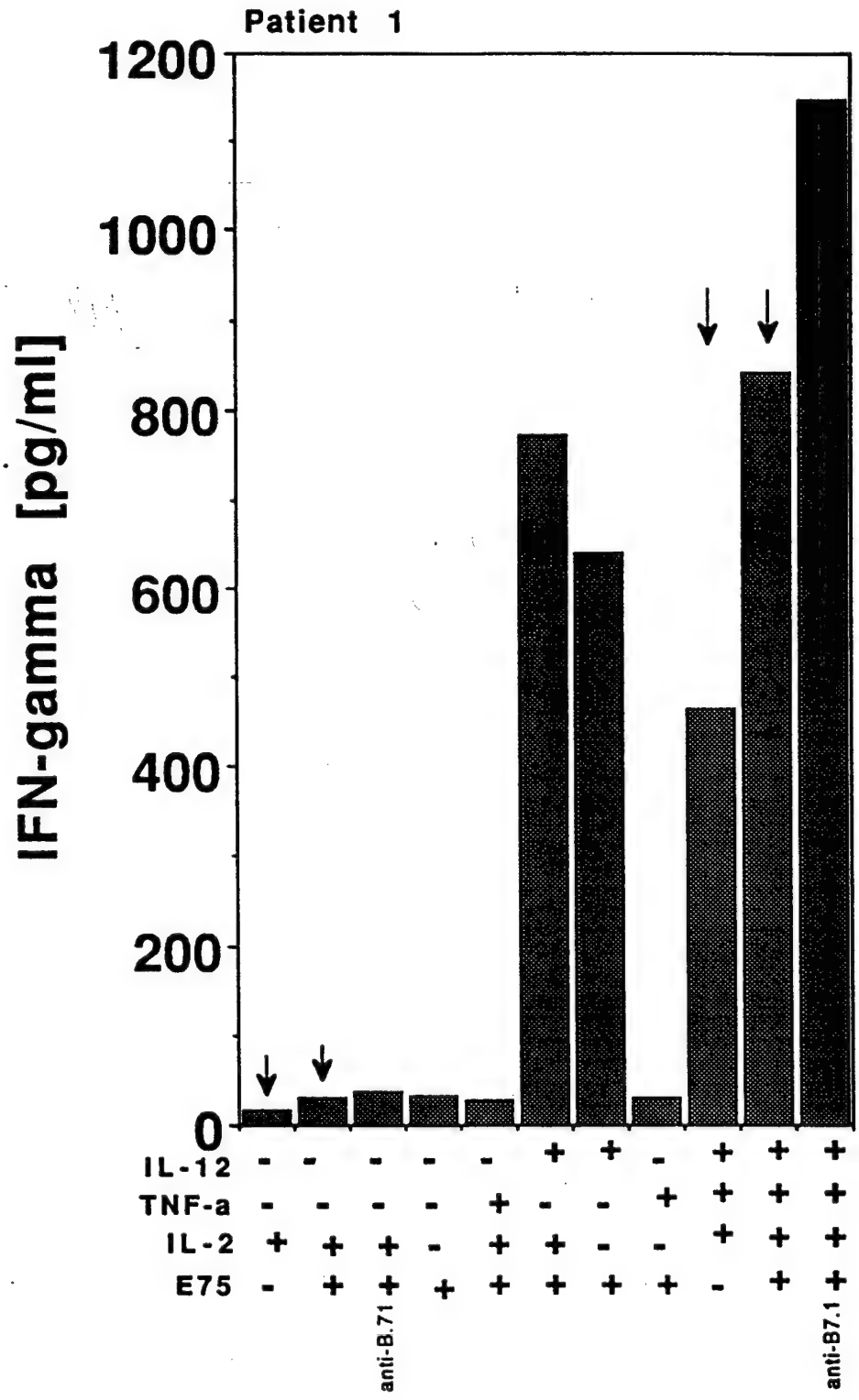

Figure 9B. Recognition of E75 by HLA.A2+ patient PBMC results in IFN-gamma secretion. IL-12 enhances this secretion.

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Mary Ann Liebert, Inc.

Growth and Antigen Recognition of Tumor-Infiltrating Lymphocytes from Human Breast Cancer

J. MICHAEL HUDSON,[1,6] AGAPITO CASTILLEJA,[1] JAMES L. MURRAY,[2] TOSHIE HONDA,[1] ANDREZJ KUDELKA,[3] EVA SINGLETARY,[4] J. TAYLOR WHARTON,[1] and CONSTANTIN G. IOANNIDES[1,5]

ABSTRACT

In the present study, we isolated tumor-infiltrating lymphocytes (TIL) from 21 primary solid tumors and tumor-associated lymphocytes (TAL) from 9 malignant effusions, respectively, of breast cancer patients. Significant proliferation and expansion of T cells was observed in 23 of 30 distinct samples. TIL were isolated from primary tumors by either enzymatic digestion or mechanical disruption. The TIL cultures were initiated using OKT3 mAb in the presence of moderate concentrations (25–50 U/ml) of IL-2, followed by 100 U/ml of tumor necrosis factor (TNF)-α. TAL were not stimulated with OKT3 mAb, but all were successfully expanded in culture in the presence of IL-2 alone or together with TNF-α. Seven of nine distinct TAL grew in culture as predominantly $CD4^+$ lines. In contrast, only 14 of 21 (66%) of primary breast TIL expanded in culture and were predominantly of $CD8^+$ phenotype. Autologous tumor lysis was observed in seven of eight cases tested. Only one of the four TIL tested and one of the four TAL tested preferentially lysed autologous tumor. HER-2 peptide E75 (369–377) was recognized by two TIL lines of the five primary TIL tested and three of the four TAL tested. This suggests that E75 may be recognized by primary breast tumors. This may be of interest in developing vaccine strategies for therapeutic management of breast cancer.

INTRODUCTION

INFILTRATION OF HUMAN TUMORS with lymphocytes is believed to indicate an immune response to tumor, as shown by a large body of studies in melanoma, renal cell carcinoma, and ovarian carcinoma. Similarly, T cells constitute the dominant leukocyte population associated with primary breast tumors. Of these, $CD8^+$ cells are present in tumor infiltration sometimes in higher percentage than in peripheral blood. Correlation between HLA-DR expression in $CD8^+$ tumor-infiltration lymphocytes (TIL) and MHC-I and MHC-II expression on the breast tumor suggests a local immune reaction that cannot expand or eradicate the primary carcinoma.[1] Strong evidence for local activation of mononuclear cells *in situ* was exemplified by expression of cytokines and cytokine genes.[2,3] Selective expression of early activation markers was found in breast TIL associated with depressed levels of interleukin-2 (IL-2) and IL-2R, suggesting that TIL may be in a relatively anergic state.[3,4] These results are indicative of the presence of antigenic molecules for T cells at the primary tumor site.

Despite the high incidence of breast cancer, there are few studies on the immune recognition and antigens of breast tumor. There are also few studies on the feasibility of propagation of TIL from primary breast tumors. This is compounded by difficulties in isolation of breast TIL from small tumors (<0.5 cm). Evidence that breast TIL can recognize autologous tumor has been reported.[5–10] Breast TIL propagated in high concentrations of IL-2 plus lymphokine-activated killer (LAK) cell supernatant infrequently exhibited preferential lysis of autologous tumor but more frequently exhibited specific Th1 cytokine secretion.[6,8–10] Reversal of T cell anergy *in vitro* using OKT3 mAb and repeated stimulation with autologous tumor in the presence of moderate concentrations of IL-2 resulted in a high proportion of tumor-specific cytotoxic T lymphocytes (CTL) in some instances.[5,8] As this approach is dependent on the availability of autologous tumor, an alternative approach to

Departments of [1]Gynecologic Oncology, [2]Bioimmunotherapy, [3]Gynecologic Medical Oncology, [4]Surgical Oncology, and [5]Immunology, M.D. Anderson Cancer Center, Houston, TX 77030.
[6]*Present address*: The Department of Head and Neck Surgery, M.D. Anderson Cancer Center, Houston, TX 77030.

breast CTL expansion is to use antigen (Ag) recognized by these breast TIL to propagate tumor-specific CTL. Using different methods, breast TIL have been isolated that recognized MUC-1, MAGE-1, and a HER-2 peptide designated GP2 (654–662).[11–13] The Ag specificities recognized by these CTL may not always be present on the autologous tumor at the time of collection, or the specific CTL frequency may be low (in these patients, the disease progresses). However, they may reflect existent responses to sensitizing Ag expressed earlier by the tumor. The identification of several CTL epitopes on HER-2[14] presented by HLA-A2 provide a model in which these questions can be addressed.

The objectives of this study were twofold: (1) to determine if breast TIL can be expanded from small primary breast tumors and (2) to determine if breast TIL and tumor-associated lymphocytes (TAL) can lyse breast tumors and recognize HER-2 peptides defining CTL epitopes. Because TIL culture in high concentrations of cytokines may lead to activation of different effector populations, such as LAK cells, and repeated stimulation with autologous or HLA-matched tumor may lead to amplification of restricted specificities present on the stimulators, we used an alternative approach for T cell propagation. This consisted of coculture of breast TIL in the initial presence of autologous tumor (when available) and OKT3 mAb and moderate concentrations of IL-2 (25–50 U/ml). We found that breast TIL from small primary tumors isolated by enzymatic digestion or only mechanical disruption could be expanded *in vitro*. HER-2 peptide, E75 (369–377) previously shown to be recognized by ovarian TAL was also recognized by a number of breast TIL and breast TAL.

MATERIALS AND METHODS

Isolation and culture of breast TIL and TAL

Fresh primary breast tumors were obtained from 21 patients. TIL were isolated after enzymatic digestion for 4 h at 37°C, followed by separation of tumor cells from lymphocytes over 75%–100% Ficoll gradients.[10] The lymphocyte-enriched fraction containing 10%–20% tumor cells was cultured in RPMI medium with 10% fetal bovine serum (FBS), 40 μg/ml gentamicin (complete RPMI), and 50 U/ml IL-2 (Cetus) in 24-well plates (Costar, Cambridge, MA). In case of recovery of a small number of cells, TILs were grown in 48-well plates (Becton-Dickinson, Franklin Lakes, NJ) in medium with IL-2 at an initial cell density of 10^6/ml. In many instances, breast TIL from primary tumors showed slow growth during the first week in culture. To facilitate their expansion, they were cultured initially on plates coated with OKT3 mAb as described[8] in complete RPMI medium supplemented with 50 U/ml IL-2 and 100 U/ml tumor necrosis factor-α (TNF-α).[15]

When the tumor specimen was small ($\leq$0.5 cm) enzymatic digestion, even for a limited period of time, led to poor recovery of TIL. These cells could not be expanded in cultures. We, therefore, used an alternative approach for breast CTL expansion. This approach is based on the use of small tissue fragments for the growth of TIL.[15] The tumor tissue was cut in pieces ($\leq$1.0 mm) that were cultured in complete RPMI medium with 25 U/ml IL-2 and 100 U/ml TNF-α for the first 16 h. Afterward, OKT3 mAb (10 ng/ml) was added to the cultures. After 2–3 days, leukocyte migration out of the tumor pieces was observed. Tumor pieces were dispersed by pipetting up and down. The mixture was allowed to settle for 30–40 sec, and the cell suspension was replated. This procedure provided better yields of recovery than enzymatic digestion and was used for TIL-10 through TIL-14. Malignant effusions were obtained from nine patients (pleural effusions in five and ascites in four patients). Isolation of TAL was performed as described.[16] Briefly, after pelleting, the cells were resuspended in serum-free medium, layered, and centrifuged over a 75%–100% Ficoll gradient. Purified lymphocytes were cultured in complete medium without OKT3 mAb. $CD8^+$ cells were isolated on specific mAb-coated plates as described.[14]

Phenotype analysis

Expression of HLA-A2 on breast TAL and TIL and of HER-2 on tumors was determined using mAb BB7.2 (American Type Culture Collection, Rockville, MD) and Ab-2, specific for the extracellular domain of HER-2 (Oncogene Science, Manhasset, NY) as described. Briefly, the cells were incubated with primary antibody for 30 min at 4°C, followed by fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Beckton Dickinson, San Jose, CA). Expression of CD3, CD4, and CD8 molecules on TIL and TAL was determined by direct immunofluorescence using OKT3, OKT4, and OKT8 mAb conjugated with FITC (Ortho Diagnostic, Raritan, NJ). Surface antigen expression was determined using a FACScan flow-cytometer (Beckton Dickinson) with a log amplifier.

Synthetic peptides

HER-2 peptides were prepared by the Synthetic Antigen Laboratory at the M.D. Anderson Cancer Center using a solid-phase method and purified by HPLC. The designation of these peptides follows that used in our previous studies: E75 (HER-2, 369–377) KIFGSLAFL; F53 based on the sequence of GP2 (HER-2, 654–662) IISAVVGIL; E90 (HER-2, 789–797); E89 (HER-2, 851–859) VLVKSPNHV; C85 (HER-2, 971–979) ELVSEFSRM. Identity of peptides was established by amino acid analysis. The purity of peptides was more than 95%.

Cytotoxicity assays

CTL activity was measured in [^{51}Cr] release assay as previously described.[14,16] Where specific lysis was low (5%–10%) in 4–5-h CTL assays, incubation of effectors with targets was continued, and the release of [^{51}Cr] was determined again the next morning. Autologous and freshly isolated allogeneic tumors as well as the breast tumor lines SKBR3 and SKBR3.A2 were used as targets. SKBR3.A2 cells express a transfected HLA-A2 gene. The original SKBR3.A2 transfectant (HLA-A, 11, B18, 40), kindly provided by Drs. Mary Disis and Martin Cheever (University of Washington, Seattle), was subjected to additional rounds of selection with the antibiotic G418 (Gibco, Life Science). The resulting cells were 100% HER-2^+, 61.6% HLA-$A2^+$, mean channel fluorescence (MCF) = 466, compared with parental SKBR3 cells, which were 5.2% HLA-$A2^+$ (MCF = 29), and control C1R.A2 cells, which were 95% HLA-$A2^+$ (MCF = 430). To study recognition of HER-2 peptides,

we used T2 cells as indicators.[16] Control cells were made with T2 cells incubated with effectors in the absence of synthetic peptides.

RESULTS

Growth and T cell phenotype of TIL and TAL from breast cancer

TIL and TAL were propagated *in vitro* with moderate concentrations of IL-2 (25–50 U/ml). Lymphocytes from both malignant effusions and solid primary tumors exhibited significant expansion in culture over time. TIL were obtained from relatively small pieces of tumor (<2 cm in diameter). After 1–2 weeks in culture, tumor cells and other nonlymphoid cells disappeared. The culture was continued as necessary to obtain lymphocytes for further studies (usually 3–4 weeks). Overall, of 14 distinct primary breast tumors processed by enzymatic digestion, 9 distinct TIL (64%) were successfully established in culture. These TIL are listed in Table 1. The doubling time for four TIL isolated by enzymatic digestion was 3 days, for one 4 days, for three 5 days, and for the remaining one >7 days. In contrast, for most TAL, the doubling times were 3 days, and only for one 4 days. The fold expansion in the first 4 weeks ranged between 40-fold and 250-fold (data not shown). A similar pattern of rapid growth was observed for TIL isolated from tumor samples that were not subjected to enzyme digestion. As we could not determine the initial number of T cells in TIL processed without enzymatic digestion, we could not establish their doubling times. The large numbers of T cells present after 2 weeks in TIL-10-13 cultures suggested a vigorous growth in four of five cultures (except TIL-14).

Table 1. Phenotypic Characteristics of Breast TIL and TAL Cultures[a]

	Origin	HLA-A2	% Positive CD4	% Positive CD8
Tumor-infiltrating lymphocytes				
1	Primary	+	8	95
2	Primary	+	4	95
3	Primary	+	4	30
4	Primary	+	75	25
5	Primary	+	30	64
6	Primary	−	69	30
7	Primary	−	81	27
8	Primary	−	12	85
9	Primary	−	33	67
Tumor-associated lymphocytes				
1	Pleural effusion	+	65	35
2	Pleural effusion	+	60	36
3	Pleural effusion	+	66	33
4	Pleural effusion	−	74	33
5	Ascites	−	45	29
6	Ascites	−	*n*[b]	48
7	Ascites	−	73	20
8	Ascites	+	13	87
9	Pleural effusion	+	*n*	72

[a]The average doubling time for TIL and TAL from breast tumors determined for weeks 2 and 3 was 3 days excepting TIL-9 and TAL-4 (4 days), TIL-4 and TIL-8 (5 days), and TIL-5 (>7 days). TAL 1–4 and 9 were obtained from pleural effusions, whereas TAL-5–8 were from ascites.

[b]*n* indicates not determined.

Table 2. Surface Phenotype and Growth Characteristics of Freshly Isolated Breast TIL Separated Without Enzyme Digestion

TIL	HLA-A2	% Positive CD4+	% Positive CD8+	Days in culture	Cells ($\times 10^6$)[a]
10	+	16	89	14	10.5
11	+	8	95	14	12.6
12	−	29	59	21	34.0
13	+	4	61	7	7.4
14	+	37	46	11	4.7

[a]Breast tumor samples <0.5 cm were cut into small pieces and cultured in complete RPMI 1640 containing 25 U/ml IL-2 and 100 U/ml TNF-α. OKT3 mAb was added to the culture after 24 h, and the culture was continued for the indicated number of days. The numbers of lymphocytes could not be determined in the original sample.

We succeeded in propagating five of seven distinct primary breast TIL isolated by mechanical disruption of the tumor, followed by OKT3 mAb (71%) (Table 2). This included TIL-12, TIL-13, and TIL-14, which could not be propagated after enzymatic digestion. Taking into consideration that enzymatic digestion results in low cell yields and may lead to significant damage of infiltrating lymphocytes, this approach may be useful for isolation of TIL from small breast samples or needle biopsies. The T cell phenotype of the TIL was determined together with the expression of HLA-A2 (Tables 1 and 2). Nine of 14 (60%) cultures were HLA-A2$^+$. Overall, CD4$^+$ T cells predominated in 3 TIL cultures (21%), whereas CD8$^+$ cells were more numerous in 9 cultures (64%). In two cultures, both CD4$^+$ and CD8$^+$ cells failed to reach the level of 50%. A different T cell phenotype was observed for breast TAL. Seven of nine proliferated as predominantly CD4$^+$ cells. Five of nine TAL samples (50%) were HLA-A2$^+$. This is in the HLA-A2 range observed in the North American population.

Target recognition by breast TIL and TAL

Autologous tumors in sufficient numbers and of sufficient purity to study target recognition were obtained from four primary breast cancers (Nos. 1, 2, 3, 4) and four patients with metastatic tumors (Nos. 1, 2, 3 and 6). Overall, one of four HLA-A2$^+$ breast TIL and one of four breast TAL tested showed preferential recognition of autologous tumors. The specificity control targets consisted primarily of the tumors autologous with TAL-1 (HLA-A2$^+$) and TAL-6 (HLA-A2$^-$), respectively, which were lysed at low levels by the autologous TIL, and the breast lines SKBR3.A2$^-$ and SKBR3.A2$^+$. Only TIL-3 and TAL-3 showed preferential lysis of autologous tumors (Fig. 1). TAL-3 showed similar levels of lysis of autologous tumor and

of the SKBR3.A2 line but lower levels of lysis of HLA-A2$^-$ targets, suggesting the presence of a shared Ag between autologous tumor and SKBR3.A2 cells.

TIL-1 showed borderline lysis (≤5%) of autologous tumor at an E:T ratio of 20:1. TIL-2 showed nonspecific tumor lysis. TIL-4 lysed the autologous tumor minimally. Three other TIL (Nos. 6, 8, and 9), for which the autologous tumor was not available, were tested in the same experiment against a panel of three breast tumors and the NK target K562. TIL-6 and TIL-8 showed very high lysis of K562 cells (>50%) and low lysis of all the tumor targets (<20% at E:T = 20:1) (data not shown). We tentatively concluded that these TIL do not exhibit specific autologous tumor lysis. TIL-9 showed significantly lower lysis of K562 (<40% in the same conditions) suggesting that specific tumor lysis may be possible.

Of two breast CTL lines (TIL-4 and TAL-2) that lacked specificity for autologous tumor, TIL-4 lysed minimally the autologous tumor and marginally two breast targets (allogeneic tumor and SKBR3) but showed significantly higher lysis of both K562 and Daudi (MHC-I negative) cells, suggesting that they express an NK/LAK-like activity (Fig. 3A). TAL-2 lysed the autologous tumor (A2$^+$), the freshly isolated allogeneic tumor associated with TAL-1 (A2$^+$), and in some experiments SKBR3 marginally (Fig. 2). In contrast, these breast CTL showed higher lysis of LAK targets but not of the tumor targets.

Because these results suggest that tumor-specific CTL may be either present in low numbers or anergic or both, we examined the effect of stimulation with OKT3 mAb in the presence of IL-2 on the expression of autologous tumor lysis by TAL-1. The results are shown in Figure 3. Stimulation with OKT3 mAb of TAL-1, containing both CD4$^+$ and CD8$^+$ cells, led to a minimal increase in target lysis. OKT3 stimulation was most effective in eliciting the TAL-1 lytic function when separated CD8$^+$ cells were used (Fig. 3, d). The biggest difference in lysis between the autologous tumor and K562 was seen in the long CTL assay (20 h), indicating, as previously suggested, low-frequency tumor-specific CTL.[17]

Breast TIL and TAL recognize CTL epitopes formed by HER-2 peptides

Tumor-reactive CTL have been reported to recognize peptides derived from tumor Ag. Studies have shown that breast CTL can recognize (1) the core peptide from the MUC-1 in both MHC-restricted and nonrestricted fashion,[18] (2) the MAGE-1 melanoma Ag,[13] and (3) a HER-2 epitope defined by the peptide GP2 (654–662).[12] Because the HER-2 peptide E75 (369–377) was found to be immunodominant in ovarian cancer patients, we tested recognition of E75 by five breast TIL lines and four breast TAL lines. The results are shown in Figure 4. TIL-1 and TIL-2 significantly recognized E75. TIL-3, TIL-10, and TIL-11 did not recognize E75. This indicates that E75 is recognized by two of five CTL from primary breast tumors. TIL-10 and TIL-11, which did not recognize E75, recognized marginally E90 (HER-2, 789–797) and C85 (HER-2, 971–979) (data not shown). To address the question of whether breast TAL recognized E75, we tested recognition of this peptide by TAL-1, TAL-2, TAL-3, and TAL-9. These TAL were HLA-A2$^+$ and associated with HER-2^{hi} breast tumors. The results are shown in Figure 4. TAL-1, TAL-2, and TAL-9 recognized E75, but TAL-3 did not. These results indicate that E75 was recognized by three of four of the breast TAL lines. Overall, among five TIL and four TAL lines studied, five recognized the peptide E75, suggesting that it may represent a shared antigen in breast cancer.

Comparison of the CTL activity levels indicates that these

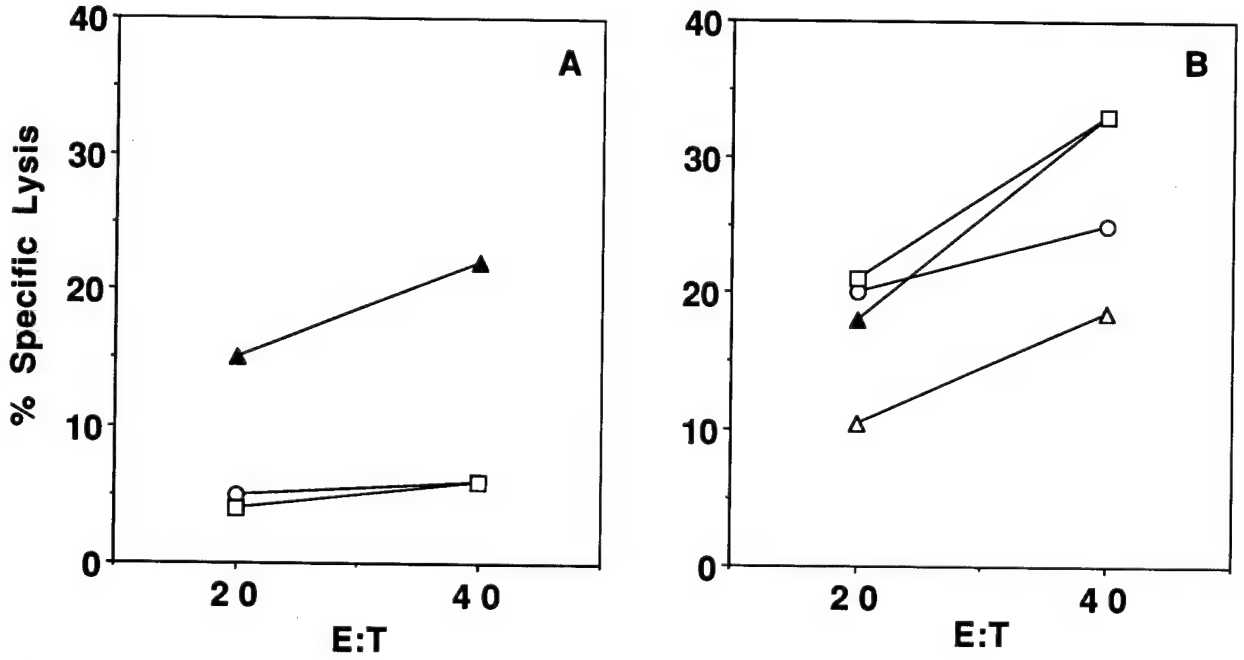

FIG. 1. Target recognition by (A) TIL-3, (B) TAL-3, (▲) autologous tumor, (△) HLA-A2$^-$ freshly isolated allogeneic tumor, (○) SKBR3 (HLA-A2$^-$), (□) SKBR3.A2 (HLA-A2$^+$). TIL-3 lysed significantly K562 cells (≥5%) only at 40:1 E:T.

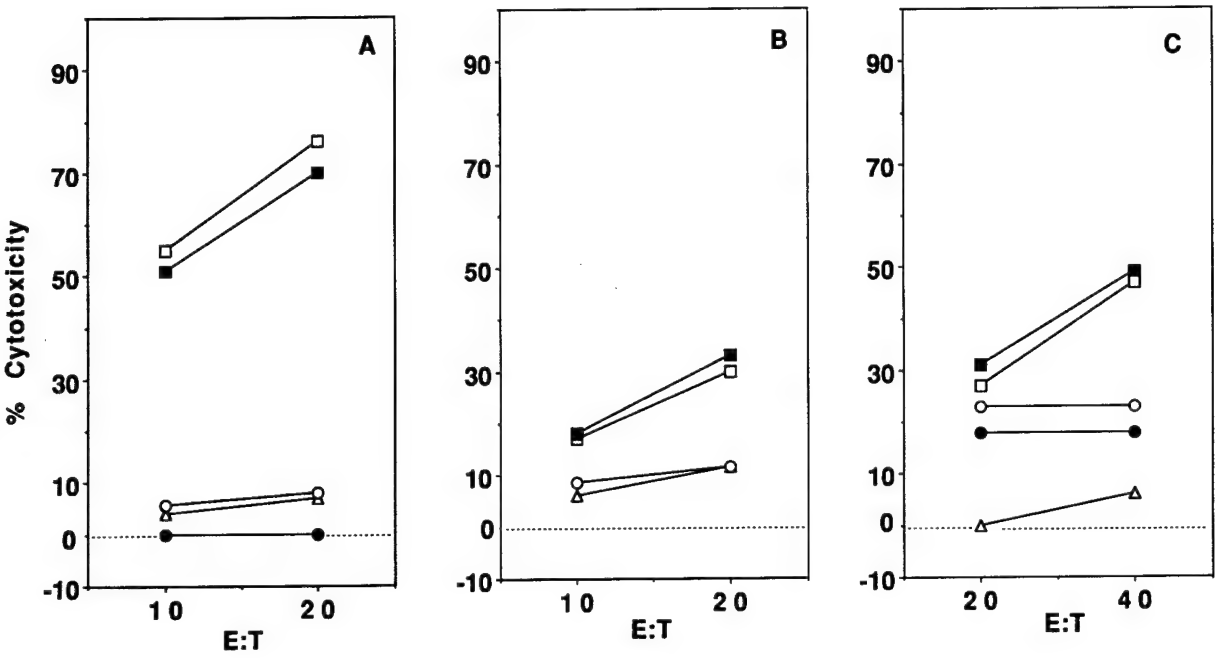

FIG. 2. (A) Cytolytic activity of TIL-4 isolated $CD8^+$ cells after 5 weeks in culture. Freshly isolated tumor (●) (□) K562, (■) Daudi, (○) tumor associated with TAL-1, (△) SKBR3. Results were determined in a 5-h [^{51}CR] release assay. Our representative experiment of two performed is shown. (B) Cytolytic activity of TAL-2 cultured for 3 weeks and stimulated with OKT3 mAb 1 week before the CTL assay. (C) TAL-2 re-stimulated with OKT3 mAb on week 3 cultured for a total of 5 weeks. (●) autologous tumor, (○) allogeneic, HLA-$A2^+$, HER-2^{hi} breast tumor, (□) K562, (■) Daudi, (△) SKBR3. The lysis of SKBR3.A2 (HLA-$A2^+$, HER-2^{hi}) was 0.0% at both 40:1 and 60:1 E:T ratios, suggesting that TAL-2 do not recognize HLA-A2-associated epitopes on this tumor.

TAL show either significantly lower recognition of autologous tumor than of HER-2 peptides (TAL-1, TAL-2) or higher specific recognition of autologous tumor but low recognition of HER-2 peptides (TAL-3). This suggests that the levels of expression of this peptide may be low and that breast CTL may recognize other Ag. Ongoing studies in our laboratory using TIL-1, TIL-2, TAL-1, and TAL-9 as indicators have identified another tumor Ag derived from the Notch receptor complex[19] (B. Babcock et al., unpublished observations).

DISCUSSION

The results presented in this study show a reproducible approach for expansion of breast TIL, with minimal intervention in terms of enzyme digestion, restimulation with autologous tumor/OKT3 mAb, and addition of exogenous cytokines. The nonenzymatic approach is complementary to enzymatic digestion. In fact, TIL-12, TIL-13, and TIL-14 were isolated from samples subjected in parallel to enzymatic and nonenzymatic processing, of which only the latter was successful. We succeeded in expanding in culture breast TIL from 14 patients and TAL from 9 additional patients. Of the established TIL cultures from primary tumors, 9 were propagated from TIL isolated by enzymatic digestion, and 5 were propagated without enzymatic digestion. TIL from 5 additional patients isolated by enzymatic digestion and from 2 patients isolated by mechanical disruption failed to grow in culture. There was a difference in the doubling times between the TIL and TAL populations. Most breast TAL showed fast proliferation, with doubling times of 3 days. In contrast, only half of breast TIL proliferated at this rate. For TIL and TAL expansion and expression of lytic function, the availability of tumor for repeated restimulation is viewed as a critical factor.[8] We observed significant T cell proliferation in 14 of 21 cases attempted (66%), including TIL from small tumors in which restimulation with autologous tumor cells could not be performed. Our results show that breast CTL-TIL can be propagated and expanded into large numbers even from small tumor samples after initial coculture with autologous tumor and stimulation with OKT3 mAb.

Fourteen breast TIL established in culture grew as predominantly $CD4^+$ or $CD8^+$ cells. This suggests that breast CTL-TIL can be successfully propagated *in vitro* in the presence of low doses of IL-2 and TNF-α, as shown by our previous studies with ovarian TAL.[18] IL-2 plus IL-12 was less effective than IL-12 plus TNF-α in propagating breast TIL (unpublished observations). This may be useful for both adoptive cellular therapies and tumor antigen identification for cancer vaccines.

The T cell phenotype of cultured TIL showed a different picture from the T cell phenotype of TAL with regard to predominance of $CD8^+$ or $CD4^+$ cells. Nine of 14 primary TIL expanded as predominantly $CD8^+$ lines (with $\geq$50% $CD8^+$ cells in the population), whereas only 2 of 9 TAL were predominantly $CD8^+$. As both TIL and TAL were cultured under the same conditions without regard of the source, the different outcomes in the phenotypes may suggest an active priming and

stimulation *in situ* of $CD8^+$ in primary tumors. The fact that the average of $CD8^+$ in the peripheral blood ranges between 25% and 33% suggests an active recruitment of $CD8^+$ cells at the primary tumor site, compared with the pleural effusion/ascitic TAL, where contamination from passenger lymphocytes may be higher.

The limited use of stimulation with tumor and OKT3 mAb for breast TAL expansion in the presence of moderate concentration of IL-2 led to breast CTL that expressed tumor lysis with higher frequency than reported in some studies[6,9,10] but lower than in other studies.[5,8] We found similar percentages (75%) of autologous tumor lysis by either TIL (3/4) or TAL (3/4). We found preferential autologous tumor lysis in only 1 of 4 breast TIL and 1 of 4 TAL tested (25%). The criteria for definition of specificity used in this study were similar to criteria used in our previous studies in ovarian cancer.[20] However, such criteria may suffer from inherent limitations because of the presence of shared Ag, the differences in target lysability, the lack of information on the HLA phenotype, and the limited amount of autologous tumor available.

Reports using cytotoxicity studies have established the presence among breast TIL and TAL of lymphocytes specifically recognizing autologous tumors. Baxevanis et al.[5] successfully expanded breast TIL from 10 patients and TAL from 2 patients after two cycles of stimulation with autologous tumor and moderate doses of IL-2; 8 of 12 TIL showed preferential lysis of autologous tumors. Specific recognition of autologous tumor was also documented by testing for cytokine secretion.[9] Dadmarz et al.[6] reported that a number of $CD4^+$ TIL recognized autologous tumor in a MHC class II restricted fashion. Specific recognition of $CD4^+$ T cells was shown by determining tumor-specific cytokine release.[6] Linehan et al.[8] successfully expanded breast TAL using low-dose IL-2 and repeated stimulation with OKT3 mAb and tumor from six of six specimens. These TAL showed preferential lysis of the autologous tumor.

The use of autologous tumor and OKT3 stimulation only at initiation of the culture may be useful to identify dominant epitope specificities of tumor-associated CTL *in situ*. The HER-2 peptides recognized by breast TIL may reflect Ag that have been stimulatory during tumor progression but either they were not sufficiently strong to elicit a curative response or novel tumor variants were selected. These variants may not express these Ag in sufficient amounts to sensitize CTL for killing or may express other Ag. It should be mentioned that all tumor Ag reported to date are recognized with low affinity.[16,21]

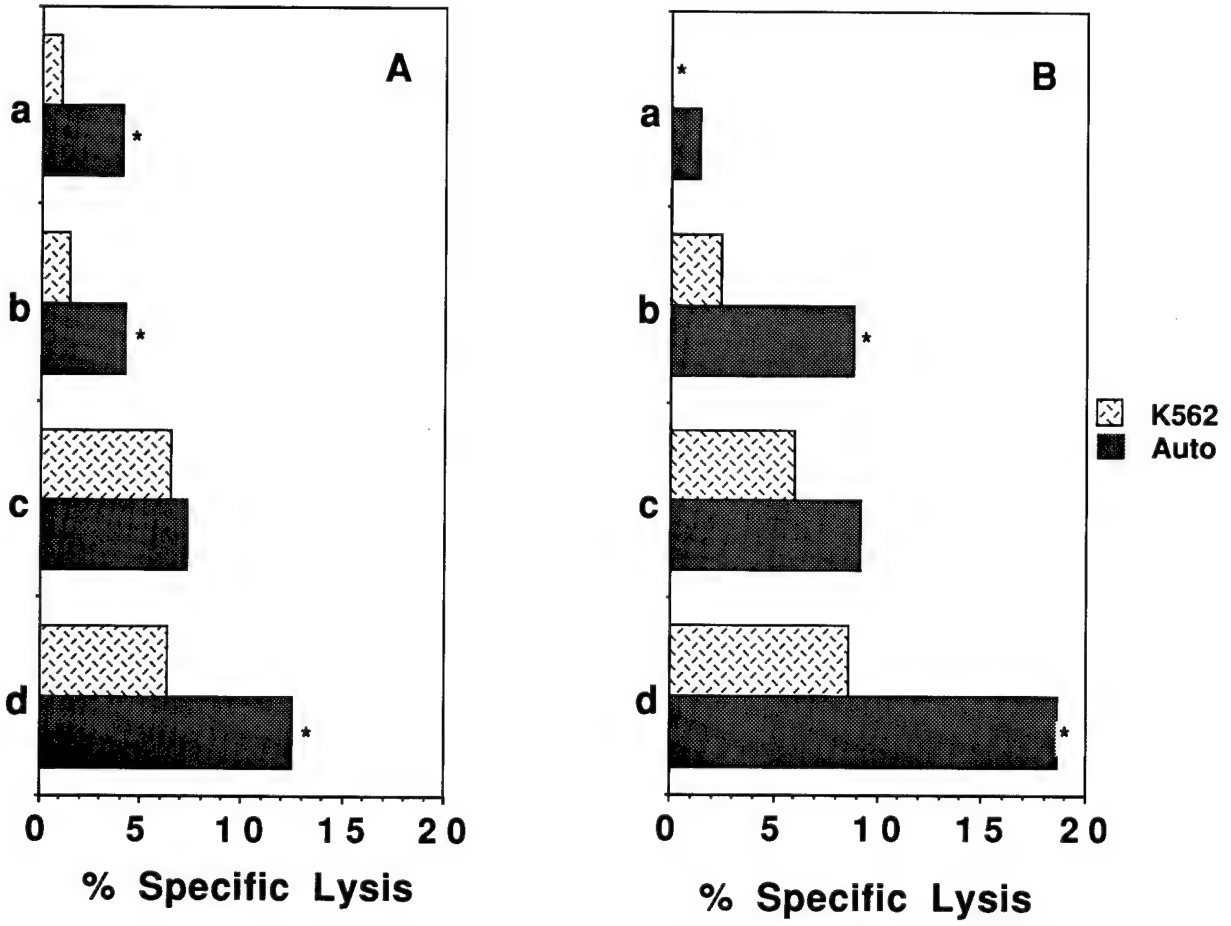

FIG. 3. Target recognition by $CD8^+$ T cells of TAL-1, stimulated in culture with OKT3 mAb. (a) Control TAL-1 bulk culture, no stimulation. (b) TAL-1 bulk culture, stimulated with OKT3 mAb. (c) Isolated $CD8^+$ cells, unstimulated. (d) Isolated $CD8^+$ cells, stimulated with OKT3 mAb. In (A) 5-h assay and (B) 20-h assay, autologous tumor and K562 cells. E:T ratio was 10:1 *Indicates that differences were statistically significant by Student's *t*-test.

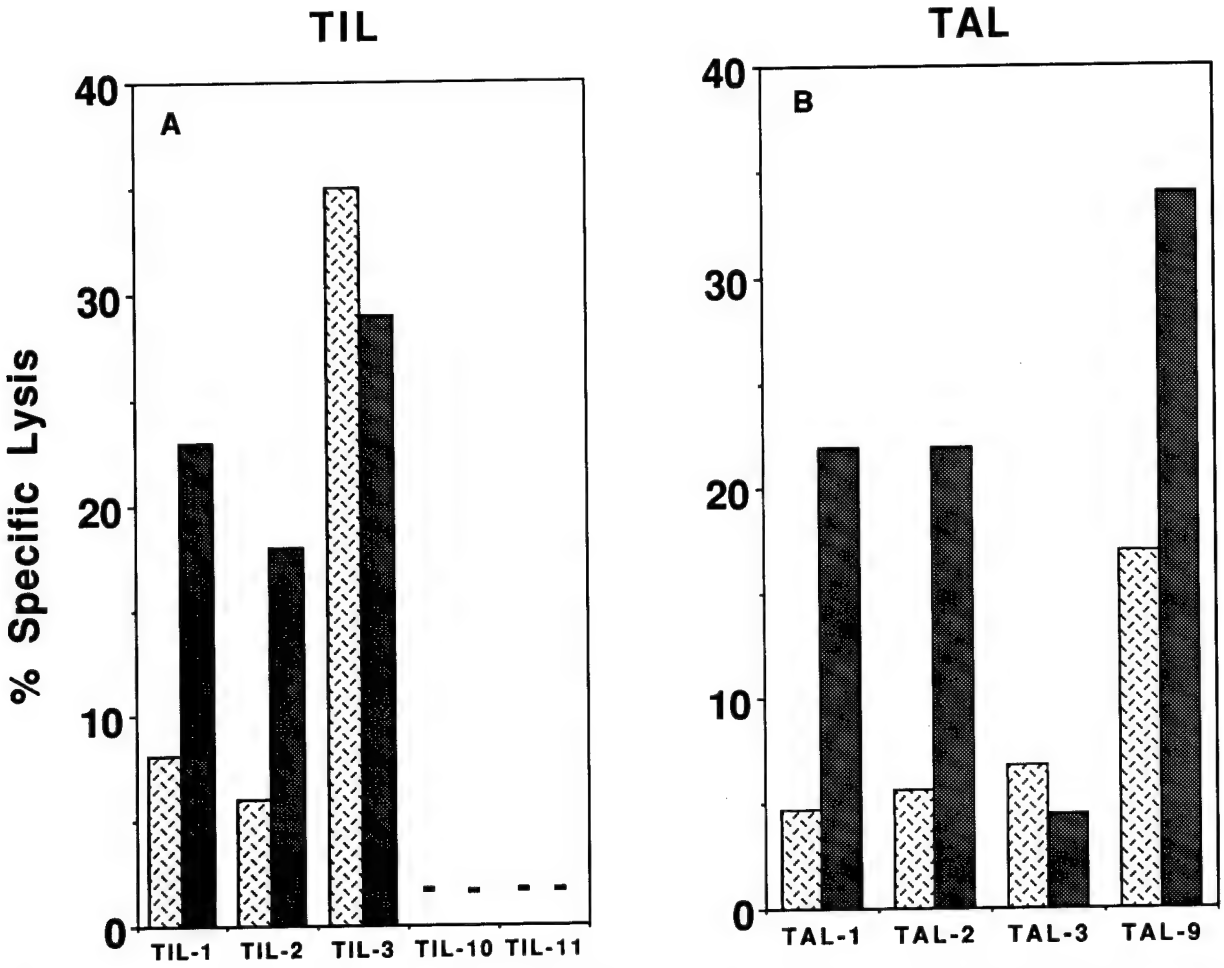

FIG. 4. Recognition by breast TIL and breast TAL of HER-2 peptides. (A) Effectors, breast TIL-10 and TIL-11, isolated from primary tumors were expanded in culture in the presence of IL-2 and initial stimulation with OKT3 mAb. E:T was 20:1. (B) Effectors were TAL-1, TAL-2, TAL-3, and TAL-9 cocultured with autologous tumor only at the initiation of the cultures. The E:T ratio was 10:1. Similar results were obtained at E:T 40:1. One representative experiment of two performed over a 4-week period is shown. Peptides were used in the assay at a concentration of 10 μg/ml. Dash indicates no lysis. (⊠) E75, (■) NP indicates that T2 cells were not pulsed with peptides before incubation with effectors.

CTL recognition of ovarian and breast tumors together with that of peptide GP2 (HER-2, 654–662) is also associated with HER-2 overexpression.[22–24] We found that HER-2 peptide E75 (369–377) is also recognized by both breast TIL and TAL. This suggests that multiple HER-2 epitopes may be presented to CTL. This raises the question of whether these CTL epitopes are always present on the tumors. TAL-1 and TAL-2 were characterized by specific recognition of several HER-2 epitopes and low and nonspecific lysis of autologous tumors. TIL-1 and TIL-2 recognized E75, but TAL-10 and TIL-11 did not recognize either E75 or F53 (GP2). TIL-3 and TAL-3 failed to recognize E75, regardless of preferential autologous tumor killing and HER-2 overexpression on autologous tumor cells. This raises several questions. (1) Do these breast CTL recognize Ag that are not present on the autologous tumors at the time of tumor resection but at an earlier stage? (2) Are the antigens present on the tumor but is their density significantly below the density required to sensitize a CTL lytic response? The T2 reconstitution assay has the advantage of amplifying the Ag density by several logs. (3) Is tumor recognition impaired by blocking molecules? The Ag may be present on the tumor, but they cannot induce a CTL response because breast tumors lack costimulatory molecules (e.g., of the B7 family). The lack of IL-2 expression in primary breast TIL has been reported recently.[4]

Conversely, if costimulatory molecules other than the B7 family are present on the tumor and can strengthen the Ag stimulatory signals, differences in Ag processing between professional antigen-presenting cells and tumor cells related to proteolytic machinery precursor stability and availability may lead to presentation of different stimulatory peptides from the same protein during tumor progression. Ongoing studies in our laboratory aim to address these points.

Identification of peptides recognized among the pool of candidate epitopes may allow development of vaccines that can be used to amplify a CTL response to breast cancer earlier, when the tumor is small. Conversely, identification of specificities amplified after repeated stimulation of TIL with autologous tumor (when available) may be useful for adoptive therapies with CTL-TIL plus cytokines. These therapies may be more suitable for patients with advanced disease, who are less likely to respond to cancer vaccination.

ACKNOWLEDGMENTS

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Address reprint requests to:
Dr. Constantin G. Ioannides
Department of Gynecologic Oncology
The University of Texas M.D. Anderson Cancer Center
1515 Holcombe Boulevard, Box 67
Houston, TX 77030

Fax: (713) 792-2849
E-mail: Ioannides_constantin@gynonc.mdacc.tmc.edu

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Potent induction of human colon cancer cell uptake of chemotherapeutic drugs by *N*-myristoylated protein kinase C-α (PKC-α) pseudosubstrate peptides through a P-glycoprotein-independent mechanism

Philip J. Bergman[1], Karen R. Gravitt[1], Nancy E. Ward[1], Pedro Beltran[1], Krishna P. Gupta[2] and Catherine A. O'Brian[1]

[1]*Department of Cell Biology, U.T.M.D. Anderson Cancer Center, Houston, Texas, USA;* [2]*Environmental Carcinogenesis Laboratory, Industrial Toxicology Research Centre, Mahatma Gandhi Marg, Lucknow, India*

Key words: protein kinase C (PKC), intrinsic drug resistance, human colon cancer, multidrug resistance (MDR), MDR reversal, *N*-myristoylated pseudosubstrate peptides

Summary

Phorbol ester protein kinase C (PKC) activators and PKC isozyme over-expression have been shown to significantly reduce intracellular accumulation of chemotherapeutic drugs, in association with the induction of multidrug resistance (MDR) in drug-sensitive cancer cells and enhancement of drug resistance in MDR cancer cells. These observations constitute solid evidence that PKC plays a significant role in the MDR phenotype of cancer cells. PKC-catalyzed phosphorylation of the drug-efflux pump P-glycoprotein was recently ruled out as a contributing factor in MDR. At present, the sole drug transport-related event that has been identified as a component of the role of PKC in MDR is PKC-induced expression of the P-glycoprotein-encoding gene *mdr1*. The objective of this study was to test the hypothesis that PKC can modulate the uptake of chemotherapeutic drugs in cancer cells independently of P-glycoprotein. We analyzed the effects of selective PKC activators/inhibitors on the uptake of radiolabelled cytotoxic drugs by cultured human colon cancer cells that lacked P-glycoprotein activity and did not express the drug efflux pump at the level of message (*mdr*1) or protein. We found that the selective PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA) significantly reduced uptake of [^{14}C] Adriamycin and [^{3}H] vincristine in human colon cancer cells devoid of P-glycoprotein activity, and that PKC-inhibitory *N*-myristoylated PKC-α pseudosubstrate synthetic peptides potently and selectively induced uptake of the cytotoxic drugs in the phorbol ester-treated and non-treated colon cancer cells. TPA treatment of the cells did not induce expression of either P-glycoprotein or its message *mdr*1. In contrast with [^{14}C]Adriamycin and [^{3}H] vincristine uptake, [^{3}H] 5-fluorouracil uptake by the cells was unaffected by TPA and reduced by the PKC-inhibitory peptides. These results indicate that PKC activation can significantly reduce the uptake of multiple cytotoxic drugs by cancer cells independently of P-glycoprotein, and that *N*-myristoylated PKC-α pseudosubstrate peptides potently and selectively induce uptake of multiple cytotoxic drugs in cultured human colon cancer cells by a novel mechanism that does not involve P-glycoprotein and may involve PKC isozyme inhibition. Thus, *N*-myristoylated PKC-α pseudosubstrate peptides may offer a basis for the development of agents that reverse intrinsic drug resistance in human colon cancer.

Introduction

In recent years, the seminal observation in 1988 that phorbol-esters induce MDR in human breast cancer cells [1] has spawned investigations into the potential role of the phorbol-ester tumor promoter receptor family protein kinase C (PKC) [2] in MDR (reviewed in [3]). In support of an integral role for PKC in MDR, phorbol-esters have been shown to induce a defect in intracellular drug accumulation accompanied by MDR in diverse cancer cell lines [1, 3–6], and increased expression of the isozyme PKC-α has been noted in a number of drug-selected MDR cancer cell lines [3, 7–9]. The report that PKC phosphorylates

P-glycoprotein [4] and the identification of the phosphorylation sites in the linker region of the drug transporter [10] led to the hypothesis that the contribution of PKC to MDR revealed by phorbol ester effects was due to PKC-catalyzed P-glycoprotein phosphorylation. This hypothesis was recently disproved by two definitive reports that characterized human P-glycoprotein mutants containing specific alterations at the phosphorylation sites. The mutations were shown to have only minor or negligible effects on the expression and transport activity of P-glycoprotein [11, 12]. The authors speculated that the contribution of PKC to MDR that is associated with altered drug transport may be restricted to induction of the *mdr1* gene, which encodes P-glycoprotein [12].

We previously reported that *N*-myristoylated PKC-α pseudosubstrate peptides partially reverse the P-glycoprotein-dependent MDR phenotype of human breast cancer MCF7-MDR cells in association with PKC-α inhibition and restoration of intracellular drug accumulation [13]. We also showed that the MCF7-MDR cells are not cross-resistant to the PKC-inhibitory peptides [13], and that the peptides are not P-glycoprotein substrates [14]. In this report, we show that phorbol-ester tumor promoters induce a significant reduction in chemotherapeutic drug uptake which is opposed by a potent induction of drug uptake by *N*-myristoylated PKC-α pseudosubstrate peptides in human colon cancer cells that do not detectably express P-glycoprotein at the level of either protein or message (*mdr*1) and do not exhibit altered drug uptake in the presence of P-glycoprotein modulators. Our results indicate that the contribution of PKC to MDR extends to effects on P-glycoprotein-independent drug transport mechanisms.

Material and methods

Materials

The metastatic human colon cancer line KM12L4a [15] was maintained as a monolayer culture as previously described [5]. This cell line has no exposure to cytotoxic drugs in its history [5, 15]. The human breast cancer lines MCF7-WT and MCF7-MDR were maintained as previously described [13]. Tissue culture reagents were purchased from GIBCO BRL (Gaithersburg, MD). The P-glycoprotein monoclonal antibody C219 was purchased from Signet Laboratories (Dedham, MA). Molecular weight markers and other SDS PAGE reagents were from BioRad Labs (Hercules, CA). The *N*-myristoylated synthetic peptides were synthesized and HPLC-purified to >98% purity at the M.D. Anderson Cancer Center Synthetic Antigen Facility, as previously described [13]. [^{14}C] Adriamycin (55 mCi/mmol), [^{3}H] vincristine sulfate (2 Ci/mmol), an enhanced chemiluminescence (ECL) detection kit, nitrocellulose sheets, and horseradish peroxidase-linked sheep anti-mouse and anti-rabbit Ig were purchased from Amersham Corp. (Arlington Hts, IL). [^{3}H] 5-fluorouracil (14 Ci/mmol) was obtained from DuPont NEN. Cyclosporin A was from Sandoz (East Hanover, NJ), and verapamil, phorbol-esters, and all other reagents were from Sigma Chem. Corp.

Drug accumulation assays

The accumulation of radiolabelled drugs in human colon cancer KM12L4a cells was measured by an established method [5, 13]. Briefly, KM12L4a cells were cultured on 24-well Costar plates (201 mm^2/well) at a density of 5×10^5 cells/well. Following a 20–24 h attachment period at 37 °C, the cells were preincubated for 30 min at 37 °C with test reagents under investigation for effects on drug accumulation (phorbol-esters, *N*-myristoylated peptides, verapamil, cyclosporin A). Next, the pretreated cells were incubated for 2 h at 37 °C with the radiolabelled cytotoxic drug (0.1 μM [^{14}C] Adriamycin, 10 nM [^{3}H] vincristine, or 10 nM [^{3}H] 5-fluorouracil) in the continued presence of the test reagent. The drug accumulation assay was terminated by rapidly washing the cells three times with ice-cold PBS, and cells were detached from the plate by a 30-min exposure to trypsin-EDTA at 37 °C. Cells were harvested, placed in vials containing 15 ml scintillation fluid, and counted [5, 13].

Western blot analysis

The level of P-glycoprotein expression in the cancer cell lines KM12L4a, MCF7-WT, and MCF7-MDR was measured by Western blotting [13]. Briefly, cells were collected in 2 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 15 mM β-mercaptoethanol, 5 mM EDTA, 5 mM EGTA, 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 200 μg/ml aprotinin and 2 mM vanadate), and sonicated for 10 sec at 4 °C. An aliquot was set aside at 4 °C as a crude fraction, and the remainder was ultracentrifuged at 4 °C for 60 mins at 35,000 rpm. The supernatant was discarded, and the pellet was re-

suspended in 1 ml of lysis buffer (membrane (mb) fraction). Protein concentration was determined with the BioRad protein assay kit (Hercules, CA). Samples were electrophoresed in a 7.5% SDS-PAGE gel, transferred to nitrocellulose, blocked, and probed with C219 monoclonal Ab in conjunction with an HRP-coupled secondary Ab as previously described [13]. Bands were detected using an enhanced chemiluminescence (ECL) system (Amersham). To assess loading efficiency, the nitrocellulose membranes were stripped in β-mercaptoethanol buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, 2% sodium dodecylsulfate) for 30 mins at room temperature, washed for 1 hr and then reblocked and probed with anti-β-actin Ab (Sigma) by the procedures described above. For identification of immunospecific bands, control Western blots that omitted the primary Ab (C219, anti-β-actin) were run in parallel.

Northern blot analysis

The level of P-glycoprotein mRNA (*mdr*1) expression in the cancer cell lines KM12L4a, MCF7-WT and MCF7-MDR was measured by Northern blot analysis. Cell lines were grown to approximately 75% confluence, and mRNA was extracted as previously described [16]. mRNA was electrophoresed on a 1% denaturing formaldehyde/agarose gel, electrotransferred to GeneScreen nylon membranes (DuPont Inc., Boston, MA), and UV cross-linked using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Membranes were pre-hybridized for 1 hr with Rapid-Hyb buffer (Amersham) and then hybridized as previously described [16]. The cDNA probes used in this analysis were a 1.38-kb EcoRI-cut fragment from clone pHDR5A corresponding to human *mdr*1 cDNA [17] and a 1.3-kb PstI gene fragment corresponding to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [18]. The probes were purified by agarose gel electrophoresis, recovered using GeneClean (Bio 101, La Jolla, CA), and radiolabelled by random primer-end labeling with [α-^{32}P]-deoxyribonucleotide triphosphate [19]. Hybridized blots were washed twice, dried, and exposed to Hyperfilm-MP autoradiography film (Amersham) for 2–24 hrs at –70 °C, and the film was developed in an automatic processor.

Statistical analysis

The data were subjected to statistical analysis with a two-tailed Student's *t* test using Microsoft software.

Results

We previously reported that phorbol esters significantly reduce cytotoxic drug accumulation (Adriamycin, vincristine, vinblastine) in human colon cancer KM12L4a cells to 50–80% of control levels in association with induction of drug resistance (2- to 3-fold increases in the IC_{50}'s of the cytotoxic drugs) [5] and activation of PKC-α [20]. As a test of whether the phorbol ester-induced reduction of drug uptake in KM12L4a cells could involve P-glycoprotein, we examined the effects of the P-glycoprotein modulators verapamil and cyclosporin A [1, 21] in this system. These P-glycoprotein-binding drugs potently inhibit the drug efflux activity of the transporter [21]. At concentrations that elicit potent P-glycoprotein inhibition, verapamil (5–25 μM) and cyclosporin A (1–4 μg/ml) failed to enhance [^{14}C] Adriamycin uptake in KM12L4a cells in the absence of phorbol esters (Figure 1). The phorbol-ester 12-O-tetradecanoylphorbol-13-acetate (TPA) (50 nM) significantly reduced [^{14}C] Adriamycin uptake in the KM12L4a cells as previously reported (Figure 1) [5]. Verapamil and cyclosporin A also failed to enhance [^{14}C] Adriamycin uptake in the phorbol-ester-treated cells (Figure 1). Consistent with these results, the immunoblot analysis shown in Figure 2 (Lanes 4, 5) revealed that KM12L4a cells did not express P-glycoprotein at a detectable level whether or not the cells were treated with TPA under the conditions employed in Figure 1. In addition, the Northern blot analysis shown in Figure 3 revealed that the TPA-treated and untreated KM12L4a cells (Lanes 1, 2) do not express P-glycoprotein mRNA (*mdr*1). To ensure that the lack of detection of *mdr*1 in KM12L4a cells in Figure 3 was not due to the 2 h exposure period, we also exposed the blot overnight and observed grossly overexposed bands in the MCF7-MDR lanes, a weak band in the MCF7-WT lane, and no band in the KM12L4a lanes (data not shown). These results indicate that the phorbol-ester-induced reduction in [^{14}C] Adriamycin uptake by KM12L4a cells occurs by a mechanism that is independent of P-glycoprotein-mediated drug transport.

In the Adriamycin-selected human breast cancer line MCF7-MDR, which expresses a P-glycoprotein-dependent MDR phenotype, the *N*-myristoylated PKC-α pseudosubstrate synthetic peptides NmFARKGALRQ (P1) and NmRFARKGALRQKNV (P3) (25–100 μM) [22] each induce a sharp increase in the uptake of P-glycoprotein substrates in association with inhibition of cellular PKC-α activity [13]. To test whether the

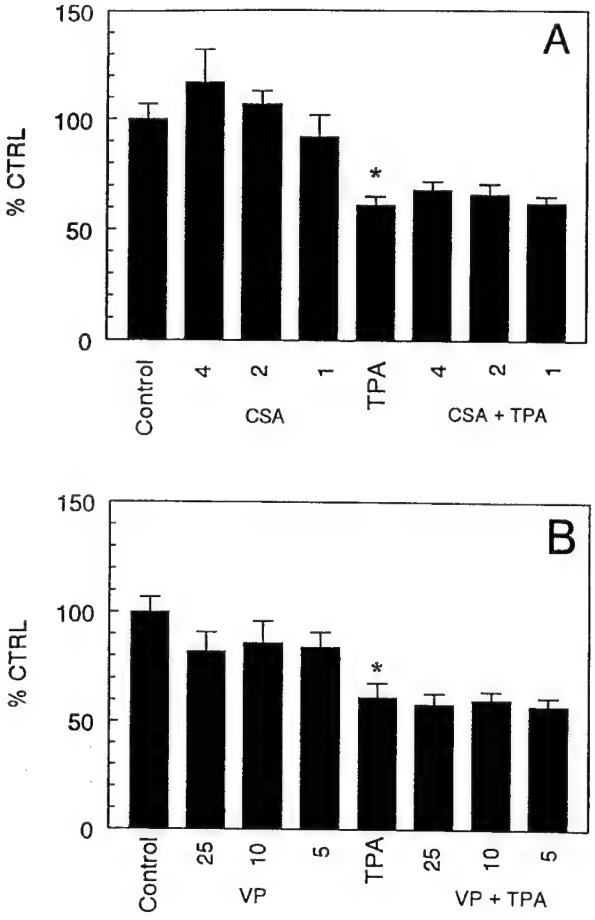

Figure 1. [^{14}C] Adriamycin accumulation in human colon cancer KM12L4a cells treated with cyclosporin A (CSA) (*A*) and verapamil (VP) (*B*) either alone (left half of each panel) or in the presence of 50 nM TPA (right half of each panel). In *A*, CSA concentrations were 0, 4, 2, and 1 μg/ml. In *B*, VP concentrations were 0, 25, 10, and 5 μM. 100% [^{14}C] Adriamycin accumulation is defined as the drug uptake measured in untreated control cells. Treatment conditions and the drug uptake assay are described in Methods. Under each treatment condition examined, >95% cell viability was observed at the end of the drug accumulation period by trypan blue exclusion. Each bar is the mean value of 3 experiments done in triplicate. * = $p < 0.01$ vs. control (untreated cells).

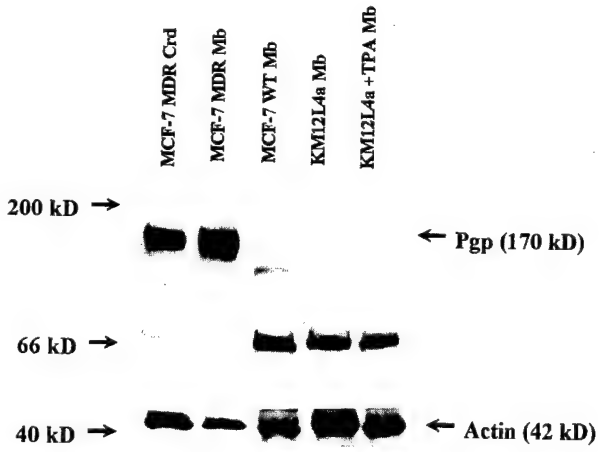

Figure 2. Immunoblot analysis of P-glycoprotein in lysates of TPA-treated and untreated KM12L4a cells is shown. The human breast cancer cell lines MCF7-MDR and MCF7-WT served as controls that, respectively, express and do not express P-glycoprotein. KM12L4a + TPA denotes a 30 min treatment of the cells with 50 nM TPA in media. Lane 1, 50 μg MCF7-MDR crude lysate protein; Lane 2, 25 μg MCF7-MDR membrane (mb) fraction; Lane 3, 25 μg MCF7-WT mb fraction; Lane 4, 50 μg untreated KM12L4a mb fraction; Lane 5, 50 μg TPA-treated KM12L4a mb fraction. The upper panel shows the result of immunoblot analysis with the P-glycoprotein-directed monoclonal Ab C219 (500 ng/ml); the bands in lanes 1 and 2 at 170 kDa are immunospecific and correspond to P-glycoprotein. The bands at 66 kDa and 130 kDa are non-specific and also appeared in control blots where C219 Ab was omitted from the analysis. The lower panel shows the result of immunoblot analysis of the same blot with β-actin Ab as a loading control. The methodology employed in the immunoblot analysis is fully described in Materials and methods. The immunoblot analysis shown is representative of the results obtained in three experiments.

effects of P1 and P3 on cytotoxic drug uptake by cancer cells extend to P-glycoprotein-independent drug transport mechanisms, we examined the effects of the pseudosubstrate peptides on drug uptake by KM12L4a cells. Figure 4A shows that P3 induced [^{14}C] Adriamycin uptake by more than two-fold over the control value in both phorbol-ester-treated and non-treated KM12L4a cells. Significant induction of [^{14}C] Adriamycin uptake was achieved at 50 and 100 μM P3 (Figure 4A). At 100 μM, P1, which is a truncated P3 analog, also achieved significant induction of [^{14}C] Adriamycin uptake in phorbol-ester-treated KM12L4a cells, resulting in a level of [^{14}C] Adriamycin intracellular accumulation that was equivalent to the level observed in control cells that were not exposed to phorbol-esters (Figure 4B). In contrast with P3, P1 (10-100 μM) did not enhance [^{14}C] Adriamycin uptake in KM12L4a cells that were not treated with phorbol-esters (Figure 4B). Thus, P3 was clearly the more potent peptide in the induction of [^{14}C] Adriamycin uptake by KM12L4a cells.

We previously reported that TPA reduces the intracellular uptake of [^{3}H] vincristine in KM12L4a cells [5]. Next, we investigated the effects of the *N*-myristoylated PKC-α pseudosubstrate synthetic peptides on [^{3}H] vincristine uptake by KM12L4a cells. Figure 5A shows that P3 significantly induced [^{3}H]

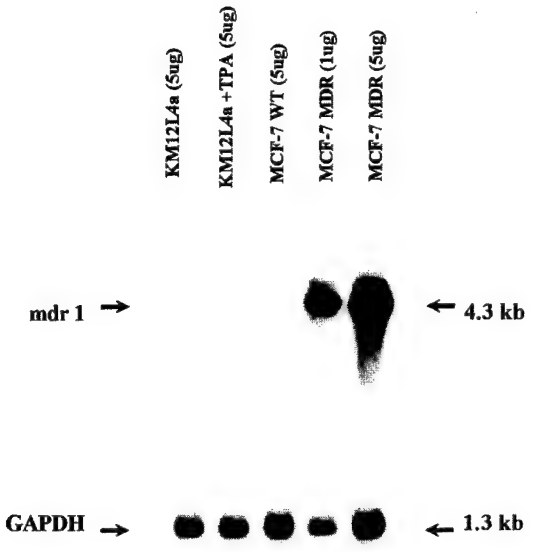

Figure 3. Northern blot analysis of *mdr*1 expression in TPA-treated and untreated KM12L4a human colon cancer cells is shown. The cell lines MCF7-MDR and MCF7-WT served as controls as described in the legend to Figure 2. mRNA was fractionated on a 1% formaldehyde/agarose gel and probed for *mdr*1. The upper panel shows the result of Northern blot analysis probing for *mdr*1 at 2 hours of exposure. The lower panel shows the result of Northern blot analysis probing for GAPDH to assess mRNA loading in the analysis shown in the upper panel. TPA treatment of KM12L4a cells was done as described in the legend to Figure 2. Lane 1, 5 μg untreated KM12L4a mRNA; Lane 2, 5 μg TPA-treated KM12L4a mRNA; Lane 3, 5 μg MCF7-WT mRNA; Lane 4, 1 μg MCF7-MDR mRNA; and Lane 5, 5 μg MCF7-MDR mRNA. Representative results of two experiments are shown. For other details, see the legend to Figure 2 and Methods.

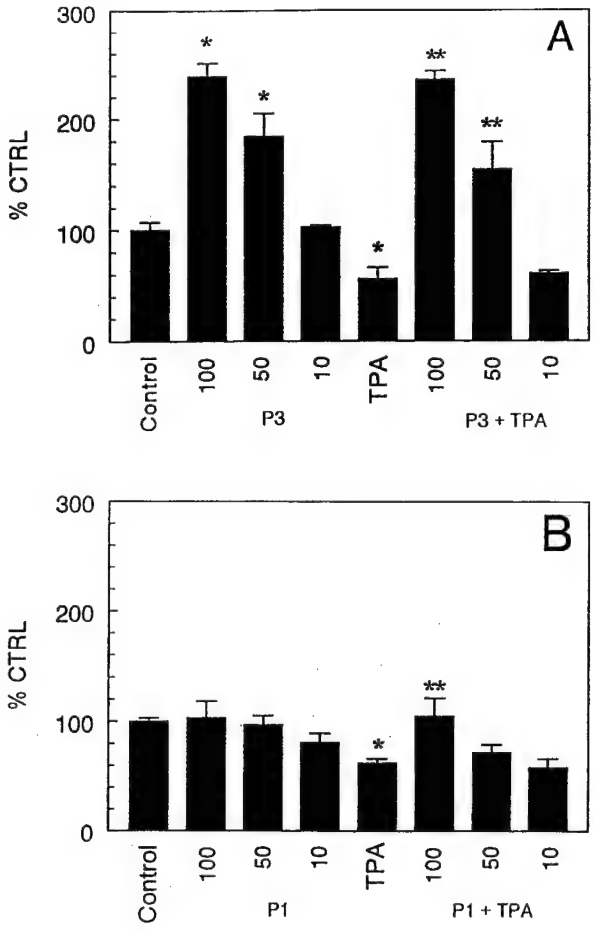

Figure 4. [^{14}C] Adriamycin accumulation in human colon cancer KM12L4a cells treated with the *N*-myristoylated PKC-α pseudosubstrate synthetic peptides P3 (*A*) and P1 (*B*) either alone (left half of each panel) or in the presence of 50 nM TPA (right half of each panel). Peptide concentrations were 0, 100, 50, and 10 μM. In *A*, each bar is the mean value of 4 experiments done in triplicate. In *B*, each bar is the mean value of 3 experiments done in triplicate. * = $p < 0.01$ vs. control (untreated cells). ** = $p < 0.01$ vs. cells treated with TPA alone. For other details, see the legend to Figure 1 and Methods.

vincristine uptake by more than four-fold over the control level in both phorbol-ester treated and non-treated KM12L4a cells. An analogous although somewhat less striking significant induction of [^{3}H] vincristine uptake was achieved in KM12L4a cells by the truncated analog P1 (Figure 5B).

In a previous report, we showed that TPA is without effect on [^{3}H] 5-fluorouracil uptake by KM12L4a cells [5]. Figure 6 confirms this and shows that neither P1 nor P3 (10-100 μM) induces [^{3}H] 5-fluorouracil uptake in phorbol-ester-treated or non-treated KM12L4a cells; in fact, the peptides appear to cause a decline in KM12L4a cell uptake of [^{3}H] 5-fluorouracil.

Discussion

Although PKC-catalyzed P-glycoprotein phosphorylation has been ruled out as a contributing factor in MDR [11, 12], the evidence that PKC activation contributes to MDR through effects on cellular drug uptake remains compelling. Most notable is the significant enhancement of MDR in association with a reduction in cellular drug uptake that is achieved in cancer cells by phorbol-ester treatment as well as by PKC-α overexpression [1, 3–6, 23, 24]. Plausible mechanisms

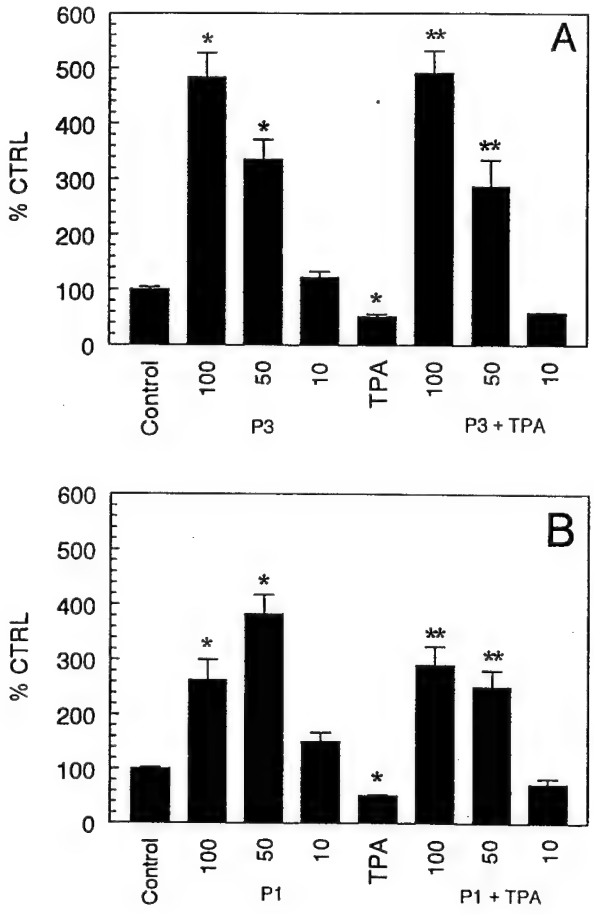

Figure 5. [^{3}H] vincristine accumulation in human colon cancer KM12L4a cells treated with *N*-myristoylated PKC-α pseudosubstrate synthetic peptides P3 (*A*) and P1 (*B*) either alone (left half of each panel) or in the presence of 50 nM TPA (right half of each panel). Peptide concentrations were 0, 100, 50, and 10 μM. 100% [^{3}H] vincristine uptake is defined as the drug uptake measured in untreated (control) cells. Each bar is the mean value of 3 experiments done in triplicate. * = $p < 0.01$ vs. control (untreated) cells. ** = $p < 0.01$ vs. cells treated with TPA alone. For other details, see the legend to Figure 1 and Methods.

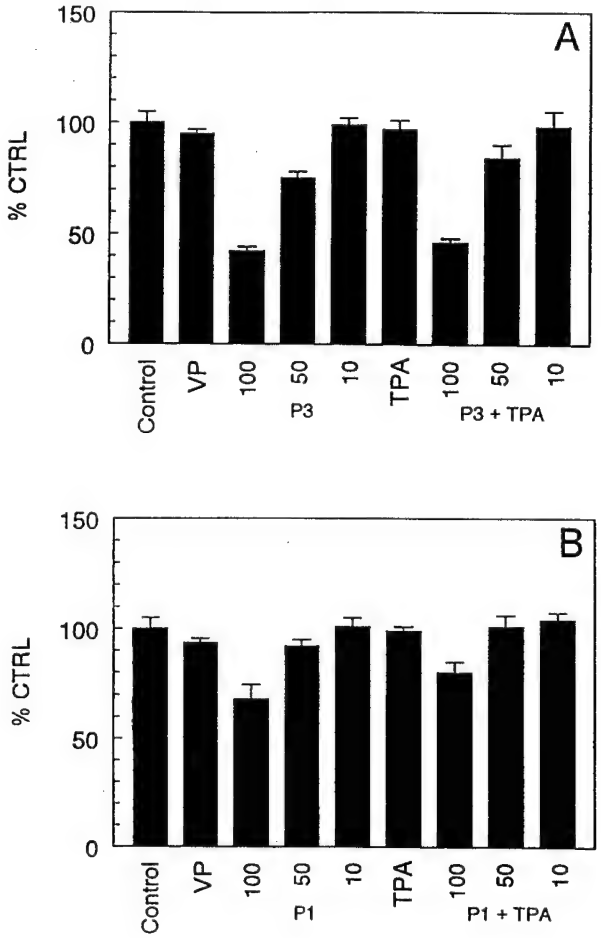

Figure 6. [^{3}H] 5-fluorouracil accumulation in human colon cancer KM12L4a cells treated with P3 (*A*) and P1 (*B*) either alone (bars to the left of TPA) or in the presence of 50 nM TPA (bars to the right of TPA). Peptide concentrations were 100, 50, and 10 μM. "Control" denotes untreated cells, "TPA" denotes cells treated with 50 nM TPA alone, and "VP" denotes cells treated with 10 μM verapamil. 100% [^{3}H] 5-fluorouracil uptake is defined as the drug uptake measured in untreated (control) cells. Each bar is the mean value of three experiments done in triplicate. For other details, see the legend to Figure 1 and Methods.

underlying the involvement of PKC in MDR by modulation of cellular drug uptake fall into three categories. 1) As previously noted [12], the role of PKC in MDR may involve *mdr*1 induction. This has, in fact, been demonstrated [25], but the extent to which this mechanism accounts for the enhancement of MDR that has been observed in association with increased PKC activity is not yet clear. 2) PKC isozymes may modulate P-glycoprotein function indirectly, by phosphorylating proteins that influence P-glycoprotein function. This mechanism would be analogous to the role of PKC in the regulation of the EGF receptor. Phorbol-esters have profound effects on the function of the EGF receptor, which is a PKC substrate [26], yet specific mutations of the EGF receptor at sites phosphorylated by PKC and MAP kinase in response to phorbol-esters appear to be without effect on EGF receptor function [27]. Recent evidence suggests that PKC may affect EGF receptor function indirectly by a mechanism involving activation of a tyrosine phosphatase [28, 29]. 3) PKC activation may enhance MDR by regulating drug transport-related mechanisms that are indepen-

dent of P-glycoprotein. In this report, we provide evidence that this is the case in the intrinsic drug resistance of human colon cancer KM12L4a cells.

Our results show that the phorbol-ester PKC activator TPA can significantly reduce drug uptake in a human colon cancer line devoid of P-glycoprotein activity and expression. Because TPA is highly selective for PKC [2], these results provide strong evidence that PKC effects on drug uptake by cancer cells include mechanisms that do not involve P-glycoprotein. Furthermore, we show that PKC-inhibitory peptides potently and selectively induce cytotoxic drug uptake in the human colon cancer line by a novel mechanism that does not involve P-glycoprotein and may involve PKC isozyme inhibition.

The intrinsic drug resistance of human colon cancer generally renders chemotherapeutic intervention ineffective in the treatment of this disease, and therapeutic interventions with biological response modifiers have not substantially improved survival [30, 31]. New strategies for the treatment of metastatic colon cancer are urgently needed [30, 31]. Our results suggest that the *N*-myristoylated PKC-α pseudosubstrate peptides described in this report may be of value in the development of novel approaches to the reversal of intrinsic drug resistance in human colon cancer.

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Address for offprints: Dr. C.A. O'Brian, Department of Cell Biology, U.T.M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 173, Houston, TX 77030, USA (Fax: 713-792-8747)

Cancer Research

American Association for Cancer Research
Public Ledger Building • Suite 826
150 South Independence Mall West
Philadelphia, PA 19106-3483
Telephone: (215) 440-9300 • Fax: (215) 440-9354

August 14, 1998

Dr. Constantin G. Ioannides
University of Texas
M.D. Anderson Cancer Center
R10.2126
1515 Holcombe Blvd.
Houston, TX 77030

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Enclosures

Increased Sensitivity of Adriamycin-Selected Tumor Lines to CTL Mediated Lysis Results in Enhanced Drug Sensitivity[1]

Bryan Fisk and Constantin G. Ioannides[2]

Departments of Gynecologic Oncology [B.F., C.G.I.] and Immunology [C.G.I.], The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030 [C.G.I.] and The University of Texas Medical School-Houston, Houston, Texas 77030 [B.F.]

Running title: CTL recognition of ADR-selected tumors

Key Words: CTL, MDR, MHC class I, Immunoselection, Chemoimmunotherapy

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[2] Please address correspondence to: Dr. Constantin G. Ioannides, The University of Texas M.D. Anderson Cancer Center, Department of Gynecologic Oncology, 1515 Holcombe Blvd., Box 67, Houston, Texas 77030, Tel: 713-792-2849, Fax: 713-792-7586.

[3] The Abbreviations used in this paper are: MDR, multi-drug resistance; TAP, transporter associated with antigen processing (TAP1 and TAP2); CTL, cytotoxic T lymphocyte; TAL, tumor-associated lymphocyte.

Abstract

The emergence of drug resistance to chemotherapeutic agents is a major cause of treatment failure in cancer therapy. Therefore, much effort has been aimed at circumventing or reversing this undesired effect. Recently, we found that tumor cell lines selected for their multi-drug resistant (MDR) phenotype can also exhibit increased levels of *TAP* mRNA and MHC class I proteins. This raised the question of whether drug resistant tumors are more readily recognized by MHC-restricted cytotoxic T lymphocytes (CTL). In this report, we show that five of five MHC I^+ tumor cell lines grown in medium containing Adriamycin developed into variants that expressed higher levels of MHC class I than their corresponding parent lines. This was not observed with a MHC I^- line. No similar association was noted for changes in expression of either HER-2 or ICAM-1 proteins. We also found that MHC class I^+ drug-selected variants were more readily lysed by MHC-restricted, tumor-associated CTL than were the drug-sensitive parent lines. When the drug-selected variants were co-cultured with the same CTL to eliminate MHC I^{hi} tumor cells, the CTL-resistant tumor cells exhibited a drug-sensitivity profile similar to that of the parent lines not exposed to adriamycin. These findings suggest that certain chemotherapeutic drugs may increase the immunogenicity of some tumors and that CTL immunotherapy may help reverse drug resistance.

Introduction

Prolonged exposure of tumor cells to cytotoxic chemotherapeutic drugs such as Adriamycin, etoposides, and vinca alkaloids, leads to the development of the MDR phenotype, which results in resistance to various types of drugs (1). The development of multi-drug resistance plays a major role in the failure of treatment of many types of cancers. Consequently, much effort has been directed at both understanding its development and deriving means to reverse or circumvent its effects. Still, this problem represents a major obstacle to progress in cancer therapy.

Two different proteins are known to mediate MDR activity: P-glycoprotein, the product of the *MDR1* gene (2), and MRP (3). These proteins are thought to act as energy (ATP)-dependent efflux pumps that prevent the intracellular accumulation of cytotoxic compounds. Both proteins belong to the ATP-binding cassette (ABC) superfamily of transmembrane transporters, the family that also includes the transporter associated with antigen processing (TAP) proteins (4-6). TAP is a heterodimer that transports peptides from the cytosol into the endoplasmic reticulum, where they are available for binding to MHC class I heavy chain (7). Such presentation of antigenic peptides is a prerequisite for the recognition and lysis of infected or transformed cells by cytotoxic T lymphocytes (CTL).

Because of the structural and functional similarities between the genes associated with multi-drug resistance and TAP, we recently investigated whether MDR tumor cells also have altered peptide transport systems (8). We found that

development of the MDR phenotype was paralleled by increased accumulation of *TAP* mRNA, resulting in a higher level of MHC class I expression, relative to the parent lines. These findings were recently confirmed by Izquierdo et al. (9), who also found both TAP and MHC class I overexpressed in several multi-drug resistant tumors.

The findings of enhanced antigen-presenting capabilities among MDR tumors raised questions about immune recognition of drug-resistant cells in comparison to their drug-sensitive counterparts. It has been demonstrated in experimental models that anticancer drugs, while often thought of as immunosuppressive, can actually potentiate a variety of immune responses (e.g. DTH, abrogation of tolerance) (10). One of the most widely studied chemotherapeutic agents in this regard is cyclophosphamide (reviewed in ref. 10). The immunopotentiation observed with cyclophosphamide is thought to result from the inhibition/depletion of suppressor T cells and may be observed with the administration of cyclophosphamide prior to tumor challenge (10,11). It has also been shown that administration of chemotherapeutic agents such as melphalan can result in increased tumor infiltration by $CD8^+$ T lymphocytes with potent, antigen-specific cytotoxic acivity *in vitro* (12). Adriamycin was found to result in a dose-dependent increase in tumor-specific CTL activity in mice receiving tumor cell vaccines, particularly when administered 1 week after vaccination as opposed to administration to vaccination (13). Furthermore, the development of regimens alternating cytotoxic therapy with immunotherapy (sequential chemoimmunotherapy) has demonstrated a synergystic effect of the two

modalities in clinical trials (14, 15). The exact mechanism by which chemotherapy induces this immunopotentiation remain to be elucidated. We hypothesized that (a) the increased expression of TAP and MHC class I proteins associated with the MDR phenotype renders such tumor cells more susceptible to recognition and lysis by MHC class I-restricted, tumor-specific CTL and (b) the elimination of the MHC^{hi} cells, within a population of multidrug-resistant cells, will result in increased sensitivity of the remaining population of cells to the cytotoxic effects of chemotherapeutic drugs by also eliminating the MDR- or MRP-overexpressing cells.

MATERIALS AND METHODS

FACS Analysis. Tumor surface antigens were detected as previously described (16), using an EPICS V Profile Analyzer (Coulter Corp., Hialeah, FL). Antibodies to HLA ABC (W6/32) (Dako, Glostrup, Denmark), HER-2/neu (Ab2) (Oncogene Science, Manhasset, NY), and ICAM-1 (Calbiochem, San Diego, CA) were not conjugated. Cells to be examined were incubated with the appropriate antibody at 4°C for 30 min, washed, and further incubated with goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN). Cells were washed again after 30 min and then analyzed.

CTL Cytotoxicity Assays. Cytotoxic activity of TAL/CTL was determined using the *in vitro* ^{51}Cr-release assay (16). CTL used as effectors were generated as previously described (16, 17). For the cytotoxicity assay, 1-2 x 10^6 target cells were labeled with 100 μCi of ^{51}Cr (Amersham, Arlington Heights, IL) at 37°C for

90 min, washed 3 times, and plated in triplicate at a final concentration of 5 x 10^3 cells/well in 96-well V-bottom microtiter plates (Costar, MA) containing the appropriate number of effector cells. For MHC class I inhibition, 5 µl of W6/32 was added to appropriate wells. Maximum release was obtained by adding 0.1 N HCL. The percentage of specific target cell lysis was determined by the following formula:

$$\frac{\text{Experimental } ^{51}\text{Cr release - Spontaneous release}}{\text{Maximum } ^{51}\text{Cr release - Spontaneous release}} \times 100\%$$

Drug Selection. Drug-selected variants were derived from breast (SKBR3, MCF-7, and MDA MB 453) and ovarian (SKOV3, MDA 2774, and CaOV3) tumor cell lines by exposure to gradually increasing concentrations of adriamycin. In brief, 1 x 10^6 cells were seeded in T-25 flasks with 12 ml of RPMI-FCS, consisting of RPMI 1640 (Gibco) + 10% FCS + 40 µg/ml gentamicin. Adriamycin (Sigma) was added at a final concentration of 1 ng/ml. Cultures were split every 3 to 4 days, at which time the Adriamycin concentration was increased. Concentrations were increased from 1 ng/ml to 2, 4, 10, 15, 20, ... to 100 ng/ml over a one month period. Adriamycin-selected tumor cells were 100% viable in 125 ng/ml adriamycin by the MTT assay. Non-selected parent lines were cultured and split simultaneously.

Immunoselection. CTL-escape variants were generated from drug-selected tumor cell lines as follows. Cells of the line to be selected were added at 5 x 10^4/ well to a 24-well Falcon plate along with 2.5 x 10^5 CTL (CTL-B or CTL-E) in a final volume of 2 ml RPMI-FCS + 50 U/ml IL-2 (Cetus). A similar number of seeded wells were incubated without IL-2 (RPMI-FCS alone) as controls. After 2 days, the

wells were rinsed gently with RPMI and the non-adherent cells removed. Both immunoselected and control wells were then further cultured in RPMI-FCS without IL-2 and split as needed. Determination of drug sensitivity was performed 7 days after initiation of selection with CTL.

Drug Sensitivity Assay. The assay was modified from that described by Wilson et al. (18). Cells were added at 2.5 x 10^4/well in a final volume of 100 µl RPMI-FCS to a 96-well flat-bottom plate, which already contained triplicate dilutions of Adriamycin. Final dilutions of Adriamycin ranged from 4 ng/ml to 500 ng/ml. Plates were incubated at 37°C for 18 h, after which 25 µl of MTT (at 2.5 mg/ml) was added per well. The plates were incubated an additional 4 h and then centrifuged for 5 min at 200x g. Medium and unconverted MTT were removed by inversion, and 75 µl DMSO added to each well. Plates were incubated on a rotator for 10 min and then read at 570 nm by a Dynatech auto plate reader. The effect of the cytotoxic drug was determined by calculating the absorbance of the test wells as a percentage of that of the control wells.

RESULTS

Adriamycin-selected tumor lines exhibit increased levels of MHC class-I. To examine the effect of chemotherapeutic resistance on the expression of tumor MHC class-I expression, we exposed six established tumor lines (3 ovarian and 3 breast) to increasing concentrations of Adriamycin. The levels of MHC class I expression of the Adriamycin-selected variants were determined then by FACS analysis and compared with the corresponding drug-sensitive parent lines. Increased levels were found in all five Adriamycin-selected variants that had

corresponding MHC class I positive parent lines **(Table 1).** The levels of increased MHC class I expression varied from a 14% increase observed with SKOV3 to 156% increase seen with MCF-7. The remaining cell lines exhibited between a 40% to 80% increase in MHC class I expression. One line, MDA 2774, consisted of two distinct populations expressing high and low levels of class I. Both populations in the Adriamycin-selected variant, 2774-DR, exhibited increased levels of MHC class I.

One parent tumor line, MDA MB453, which was negative for MHC class I expression, was used as a control. The corresponding Adriamycin-selected variant, MB453-DR, was the only drug-selected line that did not show any changes in MHC class I expression. Hence, loss of MHC class I expression is not corrected by selection with Adriamycin or the development of drug resistance.

Increased expression of the proto-oncogene HER-2 has also been described as being associated with MDR-1 over-expressing breast and ovarian tumors (19). Since increased HER-2 expression results in CTL recognition (17), we also determined the levels of HER-2 expression on the Adriamycin-selected variants. As shown in **Table 1**, HER-2 expression was slightly increased in one line, unchanged in three lines, and decreased in two other lines. We also examined for differences in ICAM-1 expression between drug-selected and non-selected tumor cells, since this adhesion molecule can facilitate tumor recognition by cellular immune effectors. Two of the six tumor lines were negative for ICAM-1 expression as were the corresponding drug-selected variants. Of the four ICAM-1 positive lines, two showed an increase of ICAM-1 expression in the drug-selected

variants, one showed no change, and one showed a slight decrease. Thus, while MHC class I expression was clearly increased in all Adriamycin-selected variants, HER-2 and ICAM-1 expression showed no such association.

Increased CTL-mediated lysis of Adriamycin-selected tumor cell lines. CTL cytotoxicity assays were performed to determine whether the increased levels of MHC class I expression in Adriamycin-selected variants resulted in increased target sensitivity to lysis. To ensure that the results were relevant for several HLA types, we used as effectors three ovarian CTL lines that express at least one HLA antigen in common with the MDA 2774 line (CTL-B: HLA-A3; CTL-E and CTL-R: HLA-A24). These CTL lines have been previously shown to preferentially lyse autologous tumors (15). All three CTL lines lysed the parent MDA 2774 cells, but higher lysis of the adriamycin-selected 2774-DR variant than of the parent line was observed **(Fig. 1A, B)**. Lysis was inhibited by the addition of the anti-MHC class I antibody W6/32 **(Fig. 1B)**. As expected, neither the MHC class I negative MDA MB453 nor MB453-DR tumors were lysed, indicating that (a) the increased sensitivity to lysis is dependent on MHC class I expression and (b) tumor lysis by these effectors is not likely to be the result of an NK-LAK activity.

A fourth CTL line, CTL-V (HLA-11, B60, 62), was tested against SKBR3 (HLA-A11, B18, 40) and its Adriamycin-selected variant SKBR3-DR. Neither SKBR3 nor SKBR3-DR was lysed by CTL-V (data not shown). Since both targets shared HLA-A11 but expressed lower levels of MHC class I than the other tumor lines tested **(Table 1),** we retested CTL-V-mediated lysis after pretreating the

tumors with 300 U/ml IFN-γ for 24 h. However, the IFN-γ treated tumors were still resistant to lysis (data not shown). These results suggest that CTL which lack antigen recognition of the Adriamycin-sensitive tumor, will not recognize the Adriamycin-selected tumor. Drug selection did not appear to alter the antigen profile of the tumor, as recognized by these effectors, but merely increased antigen presentation.

Immunoselection with CTL of adriamycin-selected variants increases drug sensitivity. On the basis of findings of increased sensitivity of Adriamycin-selected variants to CTL-mediated lysis, we hypothesized that selection by CTL may result in the elimination of those tumor cells with greater drug-resistance potential. If this hypothesis is correct then the resulting population of CTL-escape tumor variants would then be more susceptible to the cytotoxic activity of Adriamycin. To test this hypothesis, we derived CTL-escape variants by co-culturing the drug-selected lines with CTL. We then compared the Adriamycin sensitivity of the CTL-escape variants with that of both the non-CTL selected drug-resistant variants (cultured for the same interval in the absence of Adriamycin) and drug-sensitive parent lines, in MTT assays. As shown in **Figure 2A**, the non-CTL-selected 2774-DR was resistant to the cytotoxic activity of Adriamycin up to concentrations of 125 ng/ml. The parent MDA 2774 line exhibited sensitivity at Adriamycin concentrations as low as 8 ng/ml. Interestingly, the CTL-resistant variant derived from 2774-DR by selection with CTL-B exhibited an Adriamycin-sensitivity profile that was indistinguishable from the parent MDA 2774 line.

We repeated the experiment with CTL-E and the SKOV3-DR line, which share HLA-B35. Based on the MTT assay, the parent SKOV3 line appears to be more inherently resistant to Adriamycin than MDA 2774, exhibiting sensitivity at only the high concentrations of Adriamycin ($\geq$ 250 ng/ml; results not shown) similar to the profile of the drug-selected SKOV3-DR. Thus, the pattern of sensitivity to Adriamycin was in the range of 4 to 125 ng/ml **(Fig. 2B)**. This may be a reflection of prior *in vivo* selection with chemotherapeutic drugs, which is supported by the observation that among the MHC class I^+ lines, SKVO3 exhibited the lowest increase in MHC class I expression with Adriamycin exposure **(Table 1).** Of interest, the CTL-resistant variant of SKOV3-DR was sensitive to much lower concentrations of Adriamycin than were the SKOV3 and SKOV3-DR lines. Thus, our findings suggest that CTL-mediated lysis could eliminate those cells within a tumor population which are more resistant to Adriamycin, leaving a more susceptible population.

Discussion

In this report we present novel evidence that the development of resistance to chemotherapeutic agents such as Adriamycin is associated with increased susceptibility of tumors to CTL lysis. This is paralleled by an increase in MHC class I expression. Increased levels of MHC class I associated with drug selection resulted in increased sensitivity to CTL-mediated lysis. Lysis was MHC-restricted and required MHC expression. Lysis also required that the tumor presents peptide antigen to be recognized by TCR. This was suggested by the finding that

CTL-V, which could not lyse the MHC class I^+ SKBR3 parent line, was also unable to lyse the drug resistant SKBR3-DR, even after pre-treatment with IFN-γ. Therefore, the increased sensitivity to lysis of drug resistant variants seems to require both an intact antigen presentation pathway and the presence of antigen recognized by effectors.

Immunoselection by co-culture of drug-selected tumors with CTL resulted in the reversion of the surviving tumor cells to a more drug-sensitive status. This did not, however, reflect spontaneous reversion of escaping tumors to a sensitive phenotype because the same cells cultured in the same conditions without CTL were far more resistant to Adriamycin. Thus, induction of reversion of drug sensitivity is likely the result of the elimination of those tumors expressing the MDR phenotype with a concomitantly higher level of MHC class I. If exposure to a chemotherapeutic drug selects for tumor cells with increased expression of both MDR phenotype and MHC class I, it follows that after elimination of those cells the remaining population of cells will have a lower potential for expressing the MDR phenotype. The mechanisms involved in the increased TAP/MHC class I expression are not known but may involve common transcription factors and intermediates (adapter proteins) also utilized by the drug resistance genes, MDR and MRP. NF-κB has recently been demonstrated to be involved in the transcriptional regulation of genes in the *mdr* family (19) as well as regulation of TAP1 expression (21). NF-κB activity can be induced by a variety of stimuli, including cytotoxic compounds and other cellular stressors (20). The concomitant induction of stress-response NF-κB transcription factors in response

to cytotoxic stress may therefore play a role in increased expression of both *mdr* and TAP. Moreover, the Raf-1 kinase, which activates NF-κB, was also found to be involved in *mdr* expression (20,22).

It is important to emphasize that MHC class-I expression does not appear to be required for development of the drug resistant phenotype induced by Adriamycin. The absence of a requirement for MHC class I is demonstrated by the ability to select the MHC class I negative line MDA MB453 in Adriamycin. The drug-selected variant was MHC class I negative, as well. Thus, genetic changes resulting in the development of drug resistance do not seem to result in changes that can compensate for existing genetic defects in MHC class I expression. We also did not find increased expression of HER-2 and ICAM-1, making it unlikely that these play a role in the increased lysis of drug-selected tumors by CTL.

Together, our findings suggest a possible mechanism for synergy between chemotherapeutic agents and immunotherapy. Chemotherapy may decrease the tumor burden, but the remaining tumor cells, as shown here, will express increased levels of MHC class I. These remaining cells are then more likely to be recognized and eliminated by tumor-specific CTL. Furthermore, CTL may then eliminate those tumor cells with a higher potential for chemotherapeutic resistance, thus sensitizing the tumor population for a subsequent round of chemotherapeutic drug exposure. Repeated rounds of sequential chemoimmunotherapy may thus result in enhanced responses through greater reductions in tumor burdens.

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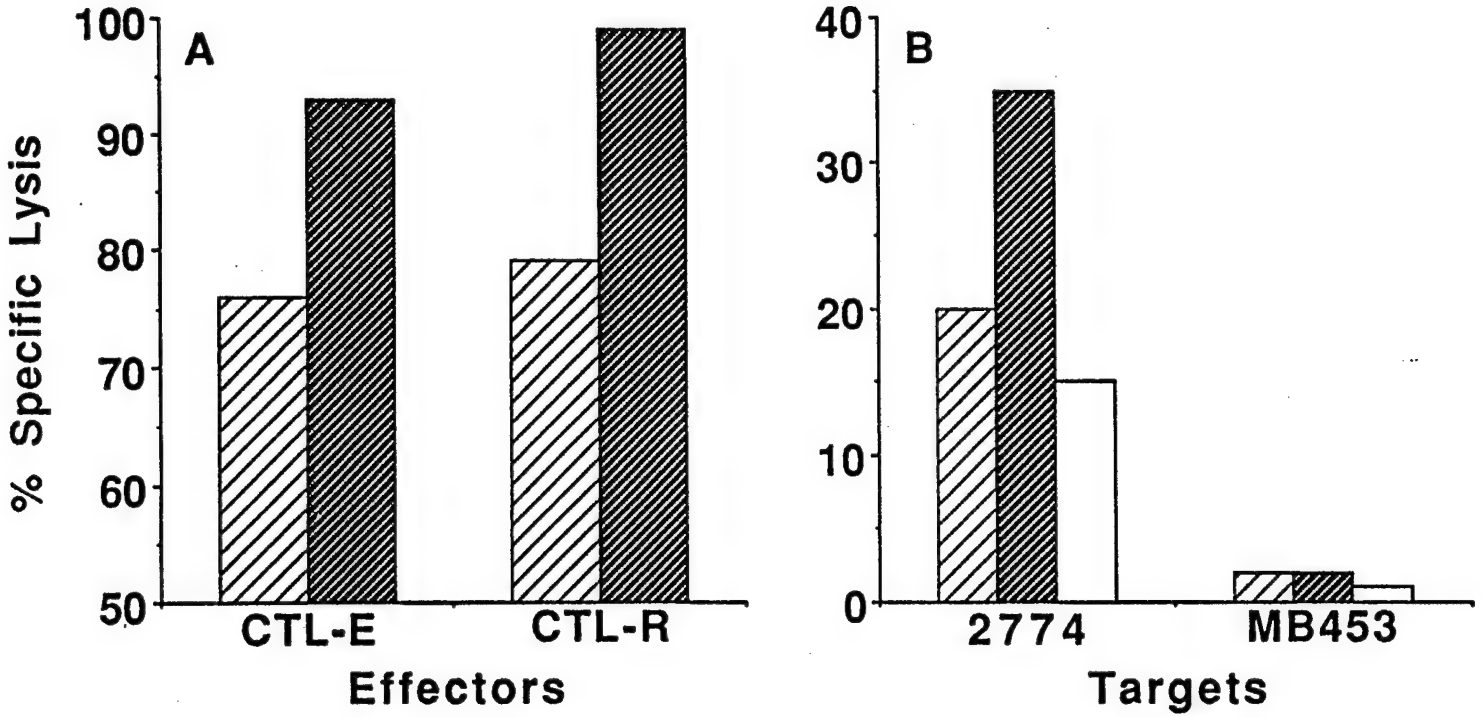
Table 1. Increases in HLA-A, B, and C Antigen Expression in Drug Resistant Tumor Cell Lines.

	Tumor cell line	Mean Level of Fluorescence: Drug-Sensitive	Drug-Resistant	MCF-R
A.	HLA Class I			
	MDA 2774	155	215	1.39
	SKOV3	190	216	1.14
	CaOV3	194	346	1.78
	MCF-7	80	205	2.56
	SKBr3	80	120	1.50
	MDA MB453	44[a]	44[a]	1.0
B.	HER-2/neu			
	MDA 2774	153	153	1.0
	SKO3	385	396	1.0
	CaOV3	10	10	1.0
	MCF-7	335	320	0.95
	SKBr3	139	139	1.0
	MDA MB453	613	536	0.87
C.	ICAM-1			
	MDA 2774	230	190	0.82
	SKOV3	111[a]	119[a]	1.27
	CaOV3	78	99	1.27
	MCF-7	275	274	1.0
	SKBr3	50	60	1.0
	MDA MB453	116	119[a]	1.0

[a] Negative samples: Antibody stained population showed no difference in comparison to negative control.

Figure 1. Increased susceptibility of the drug-selected 2774 tumor cell line to CTL-mediated lysis. CTL-E and CTL-R (A) and CTL-B (B) were tested for lysis of unselected ▨ and Adriamycin selected ▨ 2774 tumor cell lines. Additionally, CTL-B was tested against the Adriamycin-selected 2774 line in the presence of anti-MHC class I antibody, ▢ as well as against the MHC class I negative line, MDA MB453 (B).

Figure 2. Increased drug-sensitivity in CTL-resistant variants of the Adriamycin selected lines. Adriamycin-selected 2774 (A) and SKOV3 (B) were cultured in the presence ■ or absence (●) of HLA-matched CTL (CTL-B and CTL-E, respectively) for 7 days. Parent cell lines without previous Adriamycin, exposure (Δ) were cultured in identical conditions as the non-immunoselected drug-selected lines. Subsequently derived tumor lines were examined in drug-sensitivity assays as described in Materials and Methods.


A
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90
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Figure 1

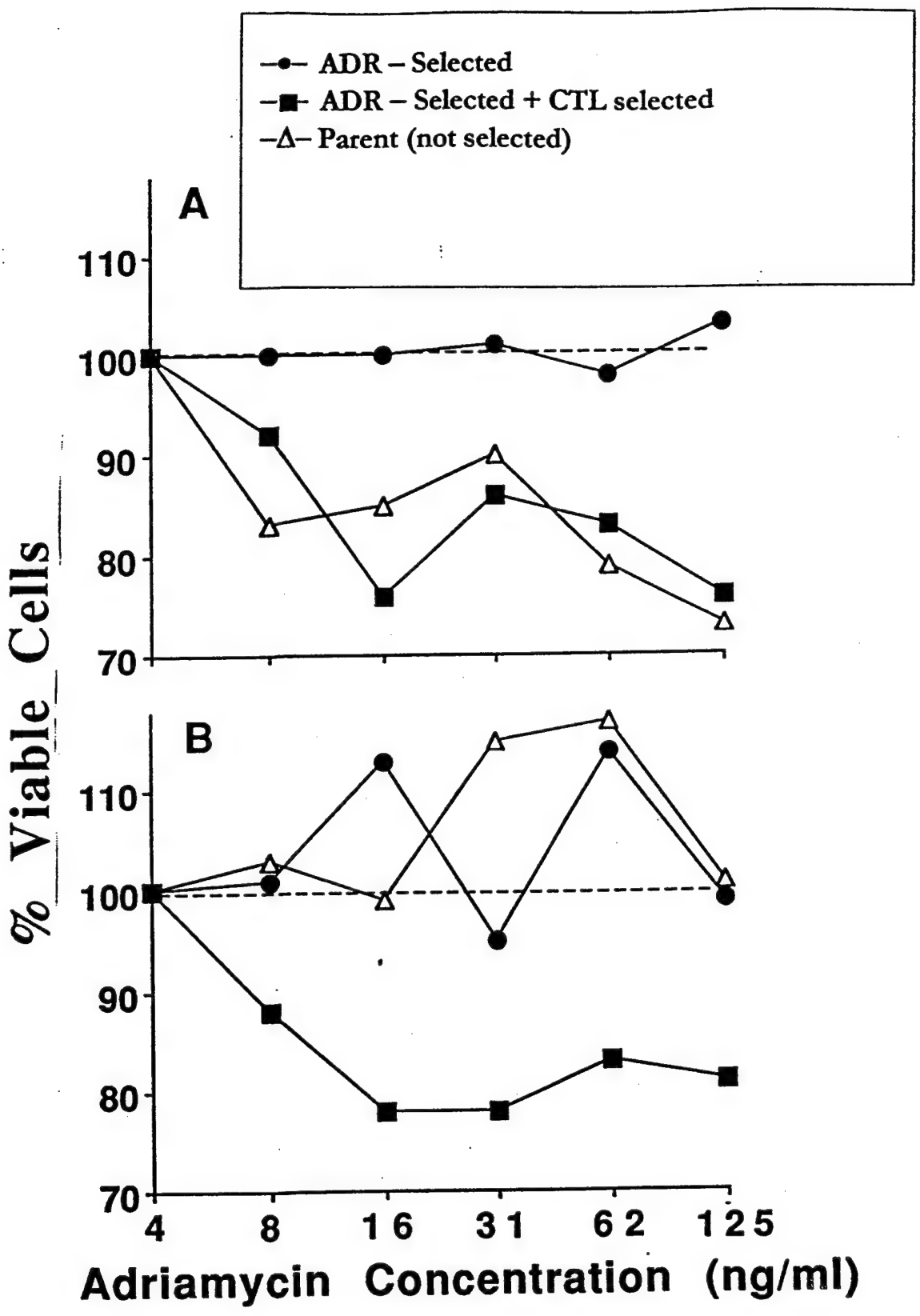
ADR – Selected
ADR – Selected + CTL selected
Parent (not selected)
A
B
110
100
90
80
70
% Viable Cells
4
8
16
31
62
125
Adriamycin Concentration (ng/ml)

Figure 2

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Dr. Constantin Ioannides
Dept. of Gynecologic Oncology - Box 67
The University of Texas, M.D. Anderson Cancer Center
1515 Holcombe Boulevard
Houston, TX 77030

Re: *" Ovarian and Breast Cytotoxic..."*

Dear Dr. Ioannides:

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[†]Ovarian And Breast Cytotoxic T Lymphocytes Can Recognize Peptides From The Amino Enhancer Of Split Protein Of The *Notch* Complex

[1]Ben Babcock, [1]Brett W. Anderson, [2]Ioannis Papayannopoulos*, [1]Agapito Castilleja, [3]James L.Murray, [4]Stefano Stifani, [5]Andrezj P. Kudelka, [1]J. Taylor Wharton, and [1,6]Constantin G. Ioannides[†]

Departments of [1]Gynecologic Oncology, [3]Bioimmunotherapy, [5]Gynecologic Medical Oncology, and [6]Immunology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, U.S.A., [2]Biogen Inc, Cambridge, MA, and [4]McGill University, Montreal Canada

Running title: CTL recognizing AES peptides.

Keywords: Notch, AES, CTL, epitopes, breast, ovary

Abbreviations: OVTAL, ovarian tumor associated lymphocytes, BRTAL, breast tumor associated lymphocytes, CID, collision-induced dissociation; AES, Amino enhancer of split; TLE, Transducin-like enhancer of split; PCR, Polymerase chain reaction; *m/z*, mass-to-charge ratio; U, mass unit; MS, mass spectrometry; w.t., wild-type; MCF, mean channel fluorescence.

[†]Please address all correspondence to: Constantin G. Ioannides, Department of Gynecologic Oncology - Box 67, The University of Texas, M.D Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, Telephone: 713-792-2849; FAX: 713-792-7586.

Abstract

In this study we investigated recognition by ovarian tumor associated lymphocyte (OVTAL) and breast tumor associated lymphocytes (BRTAL) of peptides corresponding to the sequence 125-135 of the Aminoenhancer of split (AES) protein. Three of these peptides designated as G75:AES1/2 (128-135), G60: AES1/2 (127-137) and G61: AES1/2 (125-133) correspond to the wild-type AES sequence, while the fourth G76:GPLTPLPV, AES1/2 (128-135) corresponds to a variant sequence of the peptide G75 with the N-terminal Leu substituted to glycine. These sequences were chosen for study because mass-spectrometric analysis (MS) of a CTL active HPLC peptide fraction eluted from immunoaffinity precipitated HLA-A2 molecule, revealed: *(a)* the presence of an ion with a mass-to-charge ratio (*m/z)* of 793 which was more abundant than other ions of similar masses; *(b)* the tentatively reconstituted sequence of the ion 793 matched the sequence of peptide G76. We found that AES peptides G75 (128-135) and G76 (128-135) (L128G) reconstituted CTL recognition at concentrations ranging between 200-500 nM. These concentrations are lower than concentrations reported to activate effector function of CTL recognizing other epithelial tumor Ag. Furthermore, analysis with cloned $CD8^+$ T cells indicated that G75 and G76 were not cross-reactive specificities, suggesting a key role for the N-terminal residues of the variant peptide in dictating specificities. Since the AES proteins are part of a set of transcriptional repressors encoded by the *Enhancer of split [E(spl)]* genes, and since these repressors are activated to suppress cell differentiation in response to *Notch* receptors signalling, the AES peptides may represent a novel class of self-antigens that deserve further consideration as tumor Ag in epithelial cancers.

1. Introduction

Advances in diagnostic and conventional therapies have led to earlier detection and improved quality of life for cancer patients. However, the establishment of drug resistance has raised the need for novel approaches to therapy of tumors. During recent years, studies on human cancer antigens (Ag) have identified peptides from self-proteins that are recognized by cytotoxic T lymphocytes (CTL). Most of these antigens have been discovered in the melanoma system (Houghton, 1994, Boon van der Bruggen, 1996). The expression of these CTL epitopes has been found to be dependent in some instances but not in others on the levels of MHC-class I expression (Rivoltini et. al., 1995; Fisk et. al., 1997). Regardless of the presence of CTL in tumor infiltrations, the disease progresses suggesting that this CTL response is too weak to mediate tumor regression. Furthermore, tumor progression may be dependent on an immunoselection process, characterized by the fact that tumor cells that lack expression of certain antigens may gain a proliferative advantage (Seung et. al., 1995; Kono et. al., 1997). Thus, the elimination of tumor cells expressing defined epitopes should allow unaffected growth of other tumor cells that do not express these epitopes.

Ag specific cancer vaccines may provide a complementary approach to traditional therapies if efficient targeting of cytotoxic effectors can be accomplished. In contrast with melanoma (Kawakami et. al., 1994; Castelli et. al., 1995; Cox et. al., 1994; Kawakami et. al., 1994b), there is little information on the nature of tumor Ag present on epithelial tumors such as breast and ovary which affect a large segment of the population. To this moment, the spectrum of tumor Ag and corresponding CTL epitopes in breast and ovarian cancer is limited. It includes mainly the deglycosylated Muc-1 core peptide epitope (Jerome, et. al., 1993, Ioannides et. al., 1993), and HER-2 epitopes, the latter detectable, in general, in tumors with HER-2 overexpression (Ioannides et. al., 1993; Fisk et. al., 1995; Peoples et. al.; 1995; Yoshino et. al., 1995).

Characterization of additional tumor epitopes are needed since it may allow development of polyspecific cancer vaccines, which can target a larger population of antigenically distinct tumor cells. Identification of such epitopes on epithelial tumors appear to encounter difficulties. Muc-1 and HER-2 were initially targeted for study because of their different posttranslational modification (Muc-1) or overexpression (HER-2) on tumor cells compared with normal tissues (Ioannides et. al., 1995; Peoples et. al., 1995). There is little information on other tumor genes and proteins expressed on cancer cells that can provide the focus of study of CTL recognition using synthetic peptide mapping.

An additional difficulty in characterization of novel cancer antigens rests in the limitations in the availability of primary tumor in the large amounts (>10^{10} cells) needed for biochemical characterization of extracted peptides (Cox et. al., 1994; Slingluff et. al., 1993; den Haan et. al., 1985; Udaka et. al., 1992). This leaves, at this time, as the only feasible approach for novel tumor Ag identification, the use of tumor lines as primary source of tumor peptides. Mapping of active peptide fractions from acid treated tumors using breast and ovarian CTL isolated from tumor infiltrating lymphocytes (TIL) may allow focusing the search on candidate CTL epitopes. The fact that these CTL are not induced or expanded by stimulation with the tumor line should allow identification of pre-existing epitope specificites in the patient. These peptides can then be sequenced by mass-spectometry (MS) and the candidate sequences derived from integration of resulting daughter ions tested as synthetic peptide equivalents to induce activation of CTL effector functions.

We have recently used this approach for characterization of the common peaks of naturally processed peptides shared between an ovarian tumor line (SKOV3.A2) and a freshly isolated ovarian tumor. We found in addition to a number of overlapping peaks of biological activity, several nonoverlapping peaks of activity (Fisk et. al., 1997a; Fisk et. al., 1997b). The presence of the overlapping peaks is of interest because it suggests that such epitopes may have been presented on the original primary tumor and were stimulators for CTL.

MS analysis of the ions present in the peak *B2* of overlapping activity corresponding to the HER-2 peptide E75 (Fisk et. al., 1997a, b) revealed the presence of a number of ions (Fisk et. al., 1997b). The signal intensity of several ions in a number of fractions matched the pattern of CTL activity of two ovarian tumor associated CTL-TAL lines (Fisk et. al., 1997b). One of these ions of *m/z:* 792.9 (and further designated as ion 793) was selected for sequencing by MS because its signal intensity was significantly higher than that of the other ions of similar or higher masses suggesting an abundant peptide. Reconstitution of the 793 sequence suggested several possible peptides, of which, the best match 7/8, was found within the sequence responsive amino acids 128-135 of the amino enhancer of split protein (AES-1/2) (Miyasaka et. al., 1993) of the *Notch* complex (Stifani et. al., 1992) associated with cell differentiation (Artavanis-Tsakonas et. al., 1995). Synthetic peptides of these sequences were found to reconstitute recognition of two ovarian and two breast TAL lines, isolated from ascites or pleural effusions respectively, suggesting that they may provide an additional target for tumor specific CTL.

2. Materials and Methods

2.1 *Cells and Cell Lines.*

The ovarian tumor line SKOV3.A2 has been previously described (Fisk et. al., 1995). Other targets used in these studies consisted of freshly isolated breast and ovarian tumors from malignant effusions. BRTAL and OVTAL orates or pleural effusions occurring in patients with advanced breast or ovarian carcinomas were isolated from ovarian ascites (OVA-TAL) or breast pleural effussions and ascites (BRTAL). Isolation of tumors, lymphocytes and lymphocyte culture was performed as previously described (Fisk et. al., 1995). CTL assays to determine recognition of peptide pulsed T2 cells, tumor lysis and cold-target inhibition assays followed the

previously reported procedures (Fisk et. al., 1995; Fisk et. al., 1994). Tumor peptide extraction, HPLC fractionation using two acetonitrile gradients and CTL epitope reconstitution assays have been described (Fisk et. al., 1997a, b). Effectors were generated by culture of OVTAL and BRTAL in RPMI media containing 10% FCS and 50 U/ml (Cetus) of IL-2 (complete RPMI medium).

For separation of $CD8^+$ cells, freshly isolated OVTAL and BRTAL were propagated in RPMI 1640 medium containing 10% FCS, antibiotics and 50 U/ml of IL-2 (Cetus) for one week. Afterwards the $CD8^+$ cells were isolated using magnetic beads (Dynabeads, Dynal, Oslo, Norway) and cultured with the same conditions. For the purpose of limited cloning, the $CD8^+$ cells were plated in 96_ well plates using binary dilutions ranging from 20 to 5000 cells/well in the presence of irradiated PBMC from HLA-$A2^+$ donors and alternatively stimulated with OKT3 mAb and PHA. None of these cultures was stimulated with peptides or tumor cells. Furthermore, tumor cells were not used as feeders. Two to three weeks later, the wells were scored for growth. Using this procedure, we found that in most instances proliferating cultures of $CD8^+$ cells resulted from wells initially seeded with 80 - 160 cells/well but not from wells where the $CD8^+$ cells were seeded at lower densities (Ioannides et. al., 1991). Thus we assumed that from wells containing 80 cells or more, at least one $CD8^+$ cell was able to proliferate, while such a $CD8^+$ cell was absent from wells containing half-the cell number (i.e. 40 cells) because proliferation was not observed from cultures started with 40 $CD8^+$ cells/well. Of the "clonal" cultures by the above approach, the ones that maintained stable growth for at least one month from the initiation of the limited cloning procedure were tested for peptide recognition. Since these cultures were not recloned they are designated as T cell lines.

2.2 *Mass-Spectrometry*

Five consecutive HPLC fractions (fractions 38-42) corresponding to the peak B2 of activity of peptides eluted from the immunoaffinity separated HLA-A2 molecues from SKOV3.A2 cells were analyzed by MS for the presence of ions, whose relative abundance matched the CTL activity of two ovarian CTL-TAL lines (Fisk et. al., 1997b). The single-charged ion of m/z=793 was found in fractions 40 and 41, but not in the other fractions. Identification of the ion composition of the peak B2 fractions has been recently reported (Fisk et. al., 1997b). Detailed methodological approaches to ion analysis and MS sequencing have been reported (Fisk et. al., 1997b). Sequencing of the ion 793 was performed by the Analytical Biochemistry Center of the University of Texas Medical School in Houston, Texas. Collision-induced dissociation (CID) mass-spectra were obtained with a Finnigan MAT TSQ70-triple-quadrupole instrument upgraded with TSQ700 software and a 20 kV-conversion dynode electron multiplier. For ion scans, the resolution of the first quadrupole (Q.1) was adjusted to allow transmission of +2 U from the center of the mass of interest. A peak width of 1 U was used for post-acquisition spectral averaging and quantitation by manual integration of selected chromatograms.

Sequence reconstitution was also performed using the computer program PEPSEQ version 1.2 (Sampson et. al., 1995). Although this program identifies a large number of potential candidate sequences, it also focused the search for the candidate sequence by allowing increasing stringency. Peptide sequences were identified based on the concordance of determined and predicted mass-values for peaks of ions in a candidate sequence. The concordance was determined based on the lowest deviation between experimental and theoretical values for the respective ions and defined as the lowest score/peak ratio (Fisk et. al., 1997b).

2.3 *Synthetic peptides.*

Synthetic peptides of candidate ions prepared were: G76 (AES-1: 128-135, GPLTPLPV), G75 (AES-1: 128-135, LPLTPLPV). The peptide of the same sequence

with G76 with the last two C-terminal residues inverted was designated G57: GPLTPLVP. To examine the possibility that the epitope formed is part of a longer peptide, the following peptides were prepared by extending the sequence AES-1,128-135, by two residues at C-terminus, i.e. G58: GPLTPLPVGL, G59:PLTPLVPGL, G77:GPLTPLPVGL and G78: PLTPLPVGL. To examine the possibility that the N-terminal extended sequence forms CTL epitopes two peptides were prepared G60: (AES-1, 127-135): ALPLTPLPV, and G61:(AES-1, 125-133): ALALPLTPL. The numbers assigned for the position of these peptides in the sequence follow the sequence of AES-1 protein. The sequence in the AES-2 protein is identical in this area, but the position of the sequence is N-terminally shifted by 11 residues in AES-2 compared with AES-1(21). AES-1 and AES-2 proteins, resulted likely from alternative splicing of the same precursor mRNA (Miyasaka et. al., 1993; Mallo et. al., 1995). For clarity, only the AES-1 sequence is referred to in this study. These peptides were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center and purified by HPLC to >95% purity. The codes used to identify peptide in this study were assigned by the same Laboratory. Peptides G76, G75, and G57 were sequenced by CID as described above, and their fragment ions spectra were compared with the ions resulted from the natural ion 793. To facilitate presentation, the amino acids substituted from the natural sequence or groups reverted from the natural sequence are underlined. All other peptides used in these studies have been previously described (Fisk et. al., 1995).

2.4 *Immunofluorescence.*

HLA-A2 stabilization assays were performed using the T2 line as indicator as we previously described (Fisk et. al., 1996). Expression of HLA-A2 was determined using the HLA-A2 specific mAb, BB7.2 and MA2.1. Hybridomas secreting these mAb were obtained from ATCC. The stabilizing ability of AES peptides indicative of their HLA-A2 affinity was determined from their ability to enhance HLA-A2 expression after overnight culture with T2 cells as described (Fisk et. al., 1996).

3. Results

3.1 *Characterization of the sequence of the ion 793.*

The ion 793 was sequenced by mass-spectrometry using collision induced dissociation (CID). To obtain a candidate peptide sequence, the resulting daughter ions were first examined using the program PEPSEQ version 1.2. To focus the search for a sequence, the stringency was increased after each round of analysis. At a tolerance of 0.5 U (<0.1% deviation from the mass of ion 793) only six candidate sequences were selected by the PEPSEQ program. A search in nucleic acid and protein data bases using candidate peptides containing in the sequence either Leu (L) or Ile (I) or both revealed that of the six sequences only the sequence GPLTPLPV gave the highest number of matches (7/8 matches) with a known sequence LPLTPLPV. This sequence corresponded to amino acids 128-135 of the amino enhancer of split (AES) protein. The gene for this protein is a member of the *TLE* (transducin-like enhancer of split) genes (TLE complex) associated with the *Notch* complex (Miyasaka et. al., 1993). The human AES and TLE-1,-2, and -3 proteins show significant sequence homology in other areas but differ in this particular area (e.g. human TLE-1:139-150 sequence is GPPVPLPHPSGL (Stifani et. al., 1992).

To characterize the correspondence between the experimentally determined and predicted sequence for ion 793, synthetic peptides G76: GPLTPLPV. G57:GPLTPLVP, and G75:LPLTPLPV were prepared. These peptides were then sequenced by CID. The predicted mass values for the *y* and *b* ions of peptide G76 are listed below **(Table I)**. These values were highly similar to the values predicted for peptide G57 ions, in which the last two C-terminal amino acids are reverted from the sequence of G76 (data not shown). Sequencing of peptide G76 showed a good correlation between the experimentally obtained and predicted mass values for 5/8 *b*, 4/8 *y*, 2/8 *bo*, 4/8 *yo*, and 2/8 *a*, G76 daughter ions **(Fig 1A)**. Because the amount of HPLC sample used for sequencing was limited, to integrate the sequence

the resulting daughter ion masses of the peak 793 were compared with respect the position and signal intensity with the predicted masses of the G76 daughter ions *b* and *y* and the corresponding *a*, *bo*, and *yo* ions (Papayannopoulos, 1995). The experimentally obtained sequence data for peptide G76 and ion 793 are shown side by side in **Figure 1A**. Although some major daughter ions species of peptide G76 were not detectable in the 793 spectrum, seven of eight *b* ions and four of eight *y* ions of 793 were present and found to match within 1 U, with the predicted values of the corresponding ions for peptide G76: GPLTPLPV. Furthermore, peaks corresponding to 4/8 *yo* ions, 3/8 *bo* ions and 2/8 *a* ions of the peptide G76 were also present in the spectrum of fragment ions of 793 (**Figure 1A**).

To identify a candidate peptide sequence for the ion of *m/z* 793 we used in the interpretation of the data first the candidate *b* ions, then the candidate *y* ions. Expanded CID spectra for the ion of *m/z* 793 are shown in **Figure 2A, B**. These spectra were obtained by normalizing the data to the next most abundant ions other than *m/z* 496 and 793 which were the most abundant **(Fig 1A)**. This allowed the presence and position of the smaller peaks to be determined more accurately.

From integration of the values for the candidate *b* ions *m/z* (58.2, 155.5, 369.3, 465.3, 577.0, 679.1 and 775.2) the candidate sequence appears as GPZZPLVP, (Z symbolizes an unknown residue). Then we examined the candidate *y* and *yo* ions. *b* and *bo*, *y* and *yo* ions differ in mass by a molecule of water (18 U). Thus from *y/yo* ions of *m/z* 793/775, -/717, 639/621, 526/508, 328/-, 216.9/200 (?) the first three N-terminal residues can read as GPL, while the last two C-terminal residues may read PV/VP (See also candidate ion $y2^{1+}$ *m/z* 216.9 and *yo2* *m/z* 199-200. Based on the difference in mass between candidate *b4* and *b2* ions [369.3-155.5=(213.8)] if Leu or Ile is present at P3, then the P4 residue may be Thr (Papayannopoulos, 1985). Thus integration of *y* ions led again to a candidate peptide of sequence either GPLTPLPV or GPLTPLVP (Papayannopoulos, 1995). Our data do not allow for unambigous assignment of the order of the last two amino acids. Therefore, the sequence of ion 793 deduced from *b* and *y* ions also shows a

good match (7/8) with the sequence of AES-1/2 proteins in the area 128-135, initially tentatively identified using the PEPSEQ program. The signal for N-terminal Leu (mass 113) corresponding to the wild-type AES peptide was not detected in the spectrum of the ion 793 but was detectable in the spectrum of the synthetic peptide G75 suggesting that its lack of detection does not represent a limitation of the sequencing method (data not shown).

The CID spectrum of the ion 793 also shows the presence of strong signals from ions *m/z* 124, 140, 496, as well as of signals from other ions at weaker intensity. The first three ions 124, 140, 496.4 may represent impurities (likely phospholipids, unpublished observations). This is because MS/MS spectra from other ions *m/z*: 453, 609, 638, 787, 1008 and 1017 detected in this peak (20) showed a similar pattern of background interference. For example the ion 140 was found in the CID spectra of ions of *m/z* (453, 609, 638, 787, 1008, and 1017), the ion 124 was present in the CID spectra of ions *m/z* 609 and 638, while the ion 496 was present in the CID spectra of the ions of *m/z* 609, 638, and 786.

It should be noted that these peptides were isolated as mixtures from very complex biological matrices and were not expected to be pure. Ions 124 and 140 did not correspond to dipeptides. Ion 496 was the ion with the strongest intensity in the spectrum, stronger than that of any *b* and *y* ion and even stronger than that of ion 793. Some of the other ions present may indicate the presence of an additional peptidic component at lower density, e.g., ions 279/261, 479, 566/538, and 653. Thus, at this time we cannot rule out the possibility that the active fractions contain a mixture of active and inactive components of peptide nature not all of which correspond to AES sequences.

Clarification of the presence of the missing ions in 793, and certification of its peptidic nature would have required additional material which would in turn have meant growing more tumor cells on an even larger scale (>40 x 10^{10} cells). However, the important question was whether a peptide with this sequence is functional, and

the significance of its function. We hypothesized that if the proposed sequence for the ion 793 corresponds to a peptide, and this peptide is functional then it should be able to: *(a)* bind to HLA-A2 and *(b)* activate effector pathways by $CD8^+$ T cells. Therefore to establish the significance of the ion 793 and its related peptides, we decided to investigate recognition by breast and ovarian CTL of AES peptides encompassing the amino acids 125-135.

The area AES^{-1}, 125-135 includes two overlapping sequences ALPLTPLPV (127 - 135) and ALALPLTPL (125 - 133) that contain HLA-A2 binding motifs and strong P1P2 anchors. Since the proposed sequence for the ion 793 corresponded to an N-terminally mutated AES peptide of the sequence 128 - 135, we prepared in addition to G76(GPLTPLPV) and G75(LPLTPLPV), the peptides G60(AES-1 (127-135 ALPLTPLPV) which differs from G75 by the presence of N-terminal Ala, and the peptide G61 (AES-1 (125 - 133) which overlaps in part (LPLTPL) with the other three peptides. It should be noted that the *m/z* of AES-1 peptides, 128-135 (designated as G75), 127-135 (G60), and 125-133 (G61) are different and higher than 793, e.g. *m/z* of G75 = 849.1. HPLC analysis indicated that they elute with distinct retention times (data not shown). Thus it is unlikely that they are present in the same HPLC peak.

3.2 *Stabilization Assays.*

To address the question of the ability of these AES peptides to bind HLA-A2 we performed T2 stabilization assays. The results in **Table II** show that, with respect reactivity with BB7.2 mAb, the epitopes formed by peptides G76 and G75 are likely to be conformationally different (Fisk et. al., 1996). Staining with MA2.1 mAb revealed a higher stabilizing ability of HLA-A2 by G76 than did staining with BB7.2 mAb. In contrast, the levels of HLA-A2 stabilization detected by MA2.1 and BB7.2 mAb were similar for the peptide G75 corresponding to the wild-type AES-1 (128-135) sequence. The possibility that G75 and G76 are conformationally different epitopes was strengthened by the fact the peptides G77 and G78 (AES-1,

128-137) which differ from G76 and G75 by the presence of the C-terminal group Gly-Leu, induced a similar pattern of staining with G76 and G75 by MA2.1 and BB7.2 mAb. C-terminal extension of the epitope (as in peptides G77 and G78) did not increase the binding ability of the peptide to HLA-A2. The stabilizing ability of peptides G57 and G58 with the C-terminal group reverted to VP (P7-8) was lower than that of the corresponding w.t. peptides G76 and G77.

Peptides G60 (127-135) and G61 (125-133) showed significantly higher binding affinity for HLA-A2, than did all other peptides tested. The stabilizing ability of G60 was comparable with the stabilizing activity of the high-affinity HLA-A2-associated CTL epitope influenza-matrix (M:58-66) peptide. These results suggested that peptides containing the group GP at P1-2 can bind and stabilize HLA-A2 with low affinity even though they do not express the canonical HLA-A2 anchors. This also suggested that one candidate natural peptide(s) of the G76 sequence can be presented by HLA-A2.

3.3 *Recognition of AES peptides by ovarian and breast tumor reactive CTL.*

AES sequence analysis indicate that in the same area (125-135), based on HLA-A2 binding motifs, several overlapping candidate CTL epitopes (G75, G60, G61) may be present. The L->G (128) change in the AES may reflect a variant member of the AES family as reported for other Ag (den Haan et. al., 1995). This raised the question whether the w.t. peptide G75 and/or the variant peptide G76 are recognized by TAL. We decided to investigate in parallel the recognition of a w.t. candidate CTL epitope (represented by peptide G75), of the natural candidate CTL epitope (G76), and two overlapping nonamers containing canonical HLA-A2 anchors corresponding to the w.t. AES sequences 127-135 (G60) and 125-133 (G61).

Since ion 793 was found to be expressed by an ovarian tumor line, we first tested recognition of AES peptides by ovarian TAL. To determine whether any of these AES peptides are recognized by ovarian TAL (OVTAL) we first analyzed the

concentration-dependent recognition of G76 (w.t.), G76 (natural) and of the overlapping peptides G60 and G61. All these peptides shared the motif PLTPL. The effectors were generated by brief culture in medium containing IL-2 of OVTAL from HLA-A2$^+$ donors. These OVTAL were not restimulated with tumors during *in vitro* culture to avoid changes in Ag specificity. Both G75 and G76 were recognized at a concentration of 1 μg/ml by OVTAL-1 **(Figure 3A).** Of interest at 5 μg/ml G76 recognition indicated signs of saturation. Recognition of G60 and G61 was borderline and not significantly different from controls (data not shown). Since G75 and G76 differ only in the N-terminal residue we isolated CD8$^+$ lines from OVTAL-1, and tested recognition of G75, G76 as well as the lysability of SKOV3.A2 cells by these effectors. The results **(Figure 3B)** show that the line OD8 recognized G75 but not G76. This line also recognized SKOV3.A2 cells suggesting that a similar epitope with the one formed by G75 may be present in the tumor cells. Another line, OF81, isolated from the same donor recognized G76 at concentration as low as 0.2 μg/ml but did not recognized G75 **(Figure 3C)**. The line OF81 also recognized SKOV3.A2 cells.

To evaluate whether OVTAL could recognize AES peptides G60 and G61, that bind HLA-A2 with high affinity, lysis of T2 cells preincubated with each of the low A2 affinity (G75, G76) and high affinity peptides (G60, G61) was tested in the same experiment over a range of concentrations by OVTAL from a second donor (OVTAL2). Both G76 and G75 were recognized by OVTAL-2 at lower peptide concentration (5 μg/ml) better than G60 and G61 **(Figure 4A, B, C, D)**. At 50 μg/ml recognition of G75 and G76 in fact decreased. G60 and G61 were recognized by OVTAL-2 although required significantly higher concentrations (25-50 μg/ml) than G75 and G76. Taking into consideration that G60 and G61 have significantly higher affinity for HLA-A2, than G75 and G76 this suggested that the affinity of TCR for the G76-HLA-A2 and G75-HLA-A2 epitope was higher than the affinity of TCR for the G60/G61- HLA-A2 epitopes. Since G75 and G60 differ only at their N-terminal Ala, this further indicates that the epitope(s) formed by G76/G75 were preferentially recognized over G60 and G61.

Similar results were observed when G75, G76, and G61 were tested for recognition by breast BR-TAL-1. G76 was better recognized than G75 and G61. **(Figure 5A, B, C).** Therefore, the results, using two ovarian and one breast TAL lines, suggest that both octamers, the w.t. and the variant AES epitopes are recognized by TAL, with higher affinity than the overlapping nonamers G60 and G61. These results also suggest that epitope(s) recognized by the TCR of these TAL may be located closely to the N-terminal area of these peptides, since all these peptides share the hexamer LPLTPL.

3.4 *Cold-Target inhibition experiments.*

To address whether AES epitopes are present on the autologous tumor with BRTAL-1, we performed cold-target inhibition experiments. All AES peptides tested inhibited recognition of autologous tumor by more than 50% compared with T2 cells pulsed with no peptide (T2/NP) at a cold:hot ratio of 10:1. The results in **Figure 5D** also show that G76 was more effective than G75 in inhibiting recognition of the breast tumor by the autologous BR-TAL-1, suggesting that it is likely that an epitope similar to the one formed by G76 on T2 cells is expressed on autologous tumor. Autologous tumor lysis was inhibited even more efficiently than G75 and G76, by G61. It should be noted that BR-TAL-1 recognized more efficiently G75 and G76 than G61. The reasons for higher inhibition by G61 are not known, but G61 had significantly higher stabilizing ability for HLA-A2 than G75 and G76, thus it may be possible that a higher number of HLA-A2 - G61 complexes are present on T2 cells over a longer period of time. These results also indicate that a peptide with similar or cross-reactive sequence with G61 is presented by the freshly isolated metastatic breast tumor.

3.5 Both G75 and G76 specific CD8⁺ cells are present in the freshly isolated breast TAL.

The results presented above suggest that CTL specific for either G75 or G76 or both epitopes are present in the ovarian ascites and pleural effusions. To establish whether CTL specific for one of these peptides constitute a significant population, CD8+ cells were isolated from a sample of breast pleural effusion (designated BRTAL-2) one week after culture in IL-2 and were further cultured using different starting numbers. When the cells in each culture were present in sufficient numbers to allow CTL assays they were tested for recognition of peptides G75 and G76. Of the 37 cultures tested, we found two cultures (15B and 27E) for which the levels of recognition of G76 were at least two fold higher than of G75, and one culture (27F) for which the levels of recognition of G75, were at least two fold higher than of G76 **(Fig 6A, B)**. Since these CTL were isolated from cultures initially seeded between 160 cells/well (27F, 27E) and 640 cells/well (15B), this suggests that the clonal size of G75-specific and G76-specific CTL should be significant.

To determine whether in these populations G76 specific clones are present, one of the lines, designated as 27E was recloned, and retested after expansion. The results in **Fig 6C** show that the line B27E recognized G76 at both 0.1 and 1.0 μg/ml, at an E:T ratio as low as 2:1 but did not recognized G75. This confirmed the results obtained with the two ovarian TAL lines, suggesting that CD8⁺ CTL of similar affinity and specificity for G75 and G76 are present in both epithelial tumor systems.

4. Discussion

In this study we have identified a novel candidate tumor Ag recognized by CTL present in the TAL from ovarian and breast tumors. This candidate tumor Ag consists of AES protein, which is part of the Notch complex involved in signalling for

determination of cell fate during development and differentiation. The AES protein is proposed as a candidate tumor Ag based on the ability of several peptides of AES sequence to activate and inhibit the effector function of OVTAL and BRTAL.

These peptides present certain characteristics which have not previously observed in other human peptides forming CTL epitopes: *(1)* they derive from proline rich areas and contain in the sequence at least three Pro and three Leu residues; *(2)* two of these active peptides contain Pro at position 2 (P2). P2 corresponds to the main anchor position for peptide binding to HLA-A2. It is generally occupied by Leu/Ile and less frequently by Met, Ala, and Thr (Hunt et. al.; 1992), *(3)* The most active peptides containing Pro at P2 were generally recognized with higher affinity, at 10-fold lower concentrations than the corresponding overlapping nonamers, (AL)PLTPLPV containing canonical Leu (P2) and binding HLA-A2 with high affinity. *(4)* One of the active peptides recognized with high affinity by ovarian and breast CTL, G76 corresponds to a fragment of a likely variant AES protein. This epitope is characterized by a Leu->Gly change in the N-terminal residue, a change unlikely to be the result of a point mutation in the Leu codon, since no point mutations in the Leu codon can lead to the Gly codon (Stifani, S., personal communication).

In determining the recognition of the candidate natural epitope G76, we noted that its recognition peaked at 1-5 μg/ml and was inhibited at higher concentrations (25-50 μg/ml). This may reflect sometimes the presence of impurities in the HPLC purified peptides. For Pro-rich proteins, this may also reflect Ag aggregation/ dimerization; This inhibitory activity was observed, at the same concentrations with peptide G75 which differs from G76 only at the N-terminal Leu. Although these results are preliminary since they were obtained with a small panel of effectors, they indicate that existing CTL recognize G75 and mainly G76 when pulsed on T2 targets at concentrations as low as 0.2-0.5 μg/ml (range 250-600 nM). These concentrations are significantly lower than the concentrations required to sensitize targets by most other peptides recognized by CTL in epithelial cancers

(Fisk et. al., 1995) and the majority of the melanoma tumor Ag (Houghton, 1994). Therefore, the possibility that G76 specific CTL are high affinity clones which are inhibited by high Ag concentrations, in a similar way with CTL recognizing classic Ag (Alexander-Miller, et. al., 1996) may deserve further investigation since it may provide a mechanism for tumor escape.

Based on cold-target inhibition studies, we surmise, that complexes structurally similar to the one formed by the AES peptides are present on the surface of tumor cells. Ongoing studies are attempting to clarify whether all three AES peptides are simultaneously presented by the tumor and the implications of Ag presentation from peptides containing Pro at P2.

AES peptide G76: GPLTPLPV was identified after MS sequencing of an ion with *m/z* 793 present in a HPLC fraction of peptides acid-eluted from immunoaffinity separated HLA-A2 molecules from a tumor line. This fraction reconstituted the CTL effector function of two OVTAL lines (Fisk et. al., 1997a, b). The signal intensity of the ion 793 in two consecutive HPLC fractions matched the CTL activity induced by these fractions (Fisk et. al., 1997b), thus it was considered a primary candidate for sequencing. Although our results neither provide certitude that ion 793 is a peptide, nor proof that it is the only active component of the peak 793, the approach we used may be useful for other Ag characterization studies. The extensive handling of samples used for Ag identification is accompanied by substantial losses in material (sometimes up to 90%) (Udaka et. al., 1982), which need to be compensated by growing higher numbers of cells. The availability of small amounts of samples for peptide sequencing, does not allow a direct answer to the questions as to whether a particular ion present in an active peak has attached other groups (i.e. phospholipids, sugars) or even shorter peptides that may be active by themselves. In contrast, the focus on a candidate peptide and analysis of recognition of overlapping synthetic peptides may provide a rapid answer to the question whether a candidate tumor Ag, is recognized i.e. is/was immunogenic *in vivo*.

The critical tests of tumor Ag identification strategy are whether: *(a)* the candidate peptide is active in inducing effector function by T cells from the tumor environment and *(b)* the gene and its corresponding source protein are expressed by the tumor, i.e. CTL do not recognize cross-reactive species on other targets. Our results show that the AES peptides meet the criterion of activating of effector function. Ongoing studies in our laboratory have also found that peptides G75, and G76 but not G60 can induce IL-2 secretion by OVTAL, within 24h, suggesting that they can activate additional signal transduction pathways in effectors (Babcock et. al., Manuscript in preparation). Furthermore, preliminary studies in our laboratory could not unambigously establish that *(a)* peptides with the group VP instead of PV: G59, G58, G59 and *(b)* peptides with extended C-terminal: G77, G78, G58, G59 are recognized by BRTAL and OVTAL. Thus, additional studies are needed to clarify these points. In the absence, at this time, of antibodies that unequivocally recognize human AES-1/2 in tumors and healthy tissues, we cannot quantitate the AES levels. Preliminary results in our laboratory using PCR and primers and probes specific for AES-1,2 indicate that AES transcripts are present in both the ovarian SKOV3.A2 and breast SKBR3.A2 lines, and they are distinct from the transcripts of the TLE-1-4 proteins. However, since the presence of a transcript does not always indicate the abundance of a protein, additional studies are needed to address this point.

The *AES* genes (also designated Grg) mapped to human chromosome 19 (Mallo et. al., 1995), are part of the *Enhancer of split [E(spl)]* complex of genes, that also includes the similar *TLE* genes (Stifani et. al., 1992). The *E(spl)* genes form a neurogenic locus in *Drosophila* (Artavanis-Tsakonas et. al., 1995). The exact function of E(spl) proteins is not known, but it is possibile that they function as transcriptional repressors of effector genes for cell differentiation (Jarriault et. al., 1995). These repressor pathways are induced by the activation of the membrane-bound Notch receptor and signal the suppression of differentiation. It was recently reported that TLE family members are overexpressed in combinatorial ways during differentiation of mouse embryonic carcinoma cells in vitro (Yao et. al., 1998). The

role of AES and its involvement in carcinogenesis or maintenance of undifferentiated state is unknown but the mouse analog *Grg* was implicated in inhibition of gene transcription (Mallo et. al., 1995b). The AES proteins may be widely distributed in adult mice tissues (Mallo et. al., 1995a) and possibly in human tissues (Miyasaka et. al., 1993).

Four mammalian *Notch* genes (1, 2, 3, 4) have been identified (Shirayoshi et. al., 1997. They are highly conserved relative to the Drosophila *Notch* gene and appear to be important for cell differentiation and neoplasia associated translocation (Larsson et. al., 1994). Expression of Notch-1/Notch-2 proteins have been reported in adult tissues of the mouse including among others brain, thymus, spleen and lung (Ellisen et. al., 1991), as well as human bone marrow $CD34^+$ stem cells (Varnum-Finney et. al., 1998). Transcripts of human *Notch-1* are abundant in human fetal tissues while overexpression or truncation of *Notch-(Tan 1)* are important determinants of oncotropic activity (Ellisen et. al., 1991; Pear et. al., 1996).

It may be tempting to propose that: *(a)* the synthesis of AES proteins also increase in cancer cells, to maintain the undifferentiated state as reported for TLE genes (Liu et. al., 1995); *(b)* AES may be subjected to a more rapid turnover and/or interacts with another protein as recently reported for TLE proteins (Palaparti et. al., 1997) and/or is missprocessed. *(c)* Overexpression of Notch-1 and Notch-2, as well as of the proteins of the TLE complex may result in overexpression and misprocessing of AES proteins. All these possibilities have been reported to lead to CTL epitope formation (Yewdell et. al., 1996; Michalek et. al., 1993). It remains to be seen whether AES is expressed in normal tissues, and whether the CTL recognizing these peptides also lyse healthy tissues. If the origin of ion 793 as well as the wild-type AES CTL epitopes will be confirmed, this may provide a novel Ag to target in cancer vaccination studies.

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Figure Legends

Figure 1. CID spectra of the peptide G76 **(A)** and of the ion 793 **(B)**. The ions with similar positions are marked with arrows. The tentative assignment of the ions was made by comparing the experimental values with their predicted values listed in the **Table I**. The ions which differ from the predicted values by more than 1.5-2 U are indicated with the mark(?). Note the absence of *b1* and *y1* from the spectrum of peptide G76 and the presence of the strong ionizing ion 496.4 in the spectrum of the ion 793. The experimental conditions were as described in the Materials and Methods.

Figure 2. Enlarged presentations of the CID spectra of the ion 793, after leaving out **(A)** the ions of *m/z* 496.4 and **(B)** the ion of *m/z* 793.0. Arrows indicate the positions and designation of the candidate Y and B ions by comparison with the values in Table I. Note the low signal for the ions in the expected positions for *b1*, *b2*, *b3*, and the absence of *y1*, *y3*, *y4*, *y7*.

Figure 3. **(A)** Concentration dependent recognition of AES-1 peptides G75 (0) and G76 (Δ) by OVTAL-1 at an E:T ratio of 10:1. Recognition of G75 (■), G76 (▨) and of SKOV3.A2 (▨) by two $CD8^+$ lines, OD8 **(B)** and OF81 **(C)** derived from OVTAL-1. E:T was 8:1. Recognition by OVTAL-1, OD8 and OF81 was determined in separate experiments **(A)**. One of two experiments with similar results is shown. All experiments were performed in triplicate **(B)**, **(C)**. Recognition of G75 and G76 was significantly different from control, (T2/NP), no peptide pulsed T2 cells (O). Recognition of G76 by OD8 and of G76 by OF-8-1 was 0.0. ($P<0.05$) by the unpaired Student t-test. OVTAL-1 recognition of peptides G60 and G61 was borderline and not significantly different from controls, and was not tested for lines OD8 and OF-8-1.

Figure 4. Concentration dependent recognition of AES peptides G60 and G61 by OVTAL-2. T2 targets were pulsed with **(A)** G75, **(B)** G76, **(C)** G60, **(D)** G61. E:T ratio

in the 5h assay was 10:1 in all experiments. Recognition of G75, G76 and G61 was determined in the same experiment. 0, indicates T2/NP. Recognition of G60 was determined in a separate experiment performed three days later. Both G75 and G76 were recognized better than T2/NP ($p<0.05$) at 5 μg/ml. One of two experiments performed in triplicate is shown. Results indicate mean ± SD. Recognition of G60 and G61 was significantly higher than of control (T2/NP) targets only at 25 and 50 μg/ml respectively. Experimental conditions were described in Materials and Methods.

Figure 5. **(A, B, C)** Concentration dependent recognition of AES peptides G75, G76 and G61 by BRTAL-1 in a 5h assay. All determinations were performed in triplicate in the same experiment. Results respresent mean ± SD. E:T ratio was 20:1. Recognition of G75 and G76 was significantly higher than of control T2/NP (O) at 1 and 10 μg/ml. Recognition of G61 was not significantly different from control at 1 μg/ml. **(D)** Cold-target inhibition by AES peptides of the recognition of autologous tumor by BRTAL-1. All determinations were performed in the same experiment in triplicate. Results indicate mean ± SD. Because BRTAL-1 showed weak lysis of autologous tumor in 5 h assay (7.2%), the assay was continued up to 20 h to confirm the differences in inhibition of tumor lysis. T2 cells were pulsed with each peptide at 10 μg/ml. The cold:hot ratio was 10:1. The E:T ratio was 20:1 and 40:1. (Δ) NP, (■) G75, (●) G76, ( ) G61. (*) Significant inhibition of lysis was observed with G75, G76 and G61 at E:T ratio of 20:1 ($P<0.05$) but only with G61 and G75 $P<0.05$), at E:T ratio of 40:1.

Figure 6. **(A, B)** Patterns of G76 and G75 recognition by $CD8^+$ CTL isolated by limited cloning from BRTAL-2. **(A)** $CD8^+$ CTL cultures preferentially recognizing peptide G75. **(B)** $CD8^+$ CTL cultures preferentially recognizing G76. Target T2 cells were pulsed with 1 μg/ml of G75 and G76 respectively. Because of the large number of cultures to be tested, for each culture, recognition of G75 (■) or G76 (●) was determined by an initial screening in the same experiment in duplicate. All resulting cultures were tested twice for recognition of G75 and G76. Only cultures

which were confirmed in both experiments to preferentially recognize G75 or G76 were considered positive. The E:T ratio was 3:1. $CD8^+$ cells in the wells indicated as 5, 6, 7, 8 were initially seeded at 640, 320, 160 and 80 cells/well, respectively. Designations e.g., 27F indicate plate, column, row **(C)** Concentration- dependent recognition of G76 by line B27E isolated from a $CD8^+$ culture previously found in a separate experiment **(B)** to preferentially recognize G76 over G75. The E:T ratio was 2:1. (■) G75, (●) G76; **(C)** Recognition of G76 was significant compared with that of G75 at both 0.1 and 1.0 μg/ml ($p<0.05$). The results show the means and standard deviations of one CTL assay performed in triplicate.

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Figure 1

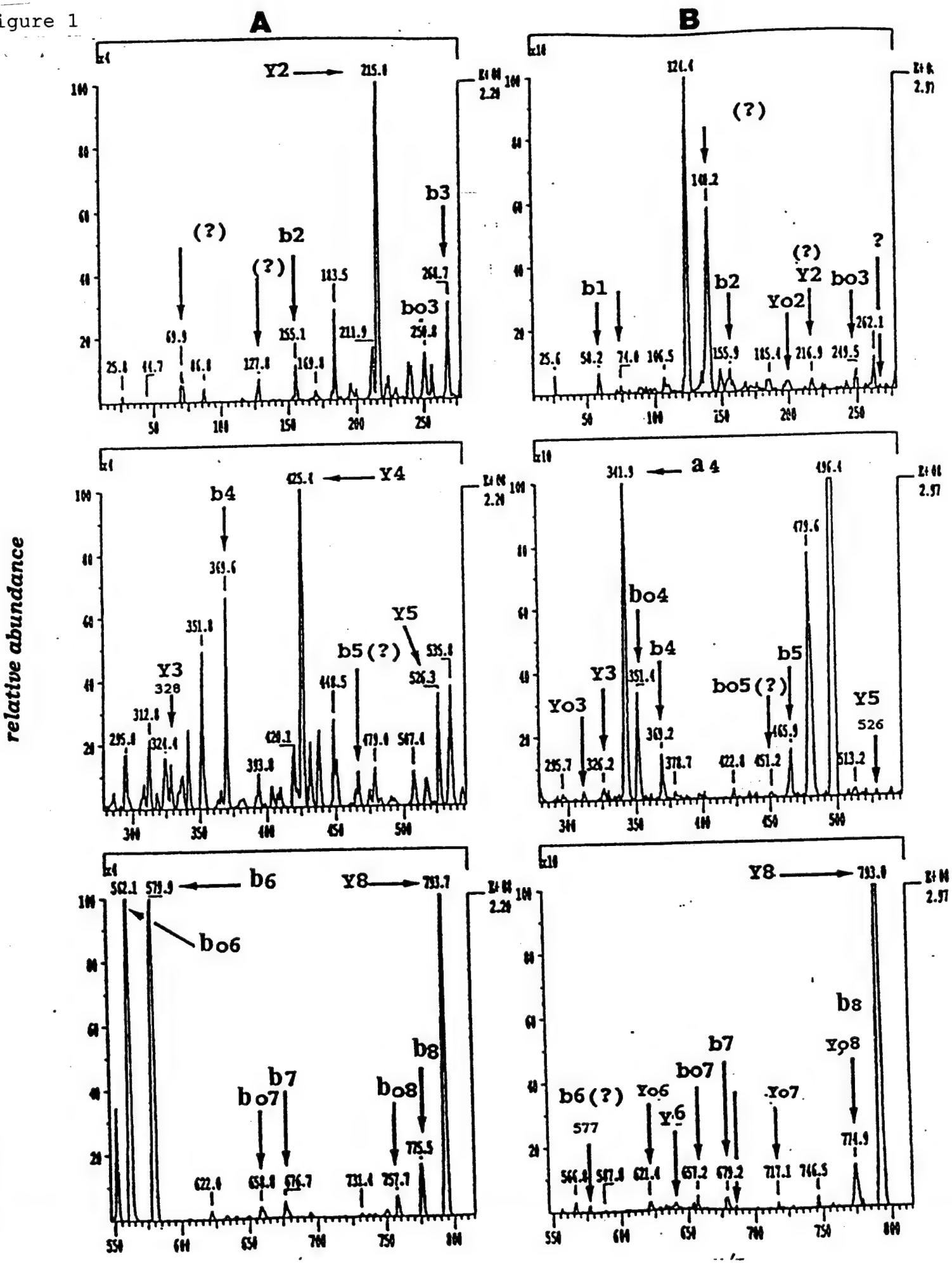
A
B
relative abundance
Y2
b3
(?)
b2
bo3
b1
Yo2
?
b4
Y4
Y5
Y3
b5(?)
a 4
bo4
bo5(?)
Yo3
b6
Y8
bo6
b7
bo7
b8
bo8
Yo6
Y6
Yo7
b6(?)
Yo8

Figure 2

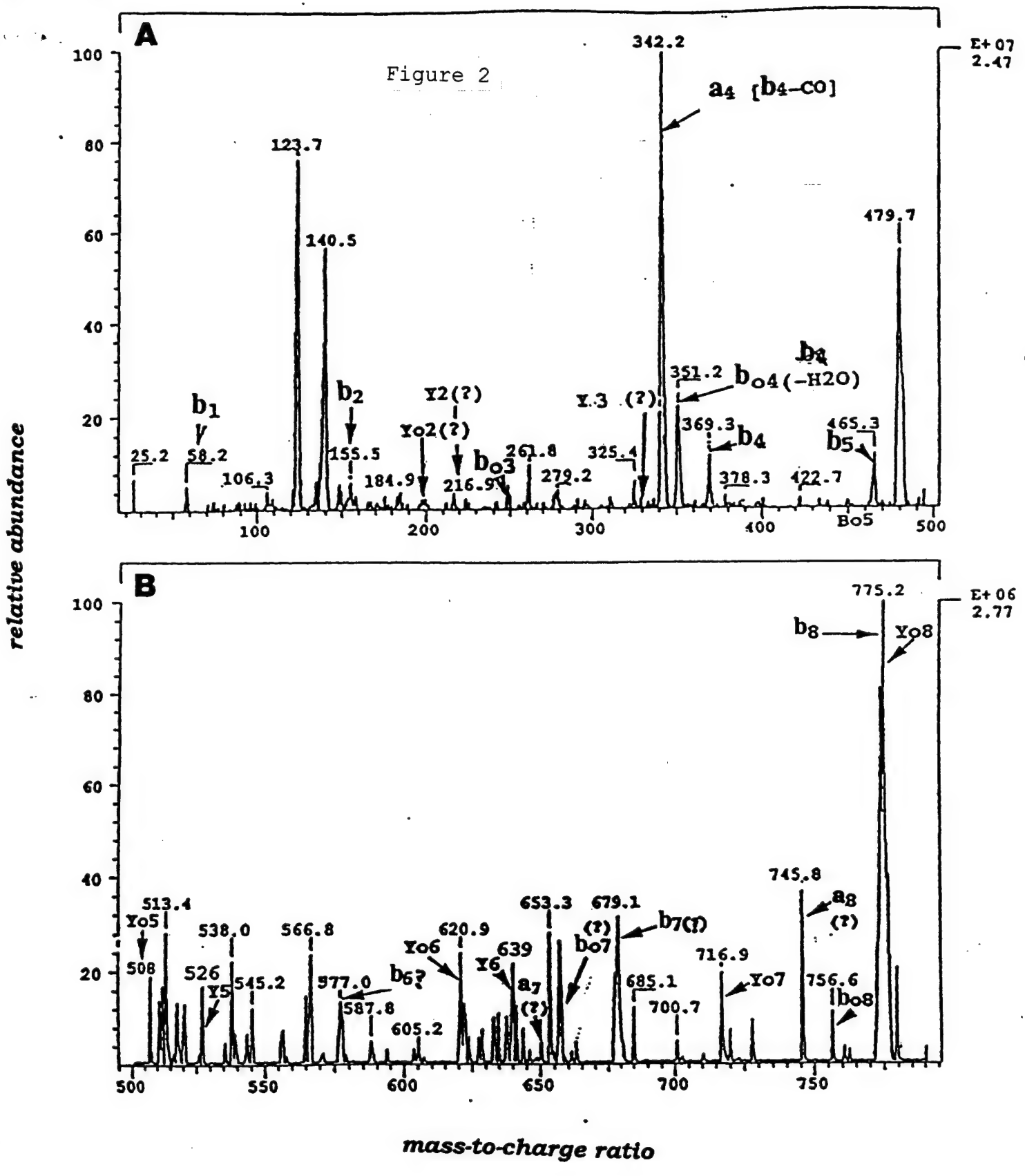
A
342.2
E+07
2.47
a4 [b4-co]
123.7
140.5
479.7
b1
b2
Y2(?)
Yo2(?)
Y3 (?)
351.2
bo4(-H2O)
b3
369.3
b4
465.3
b5
25.2
58.2
106.3
155.5
184.9
216.9
bo3
261.8
279.2
325.4
378.3
422.7
Bo5
B
775.2
E+06
2.77
b8
Yo8
513.4
Yo5
538.0
566.8
620.9
Yo6
639
Y6
653.3
679.1
(?)
b7(?)
bo7
745.8
a8
(?)
716.9
Yo7
756.6
bo8
508
526
Y5
545.2
577.0
b6?
587.8
605.2
a7
(?)
685.1
700.7
relative abundance
mass-to-charge ratio

Table I. Predicted masses of daugher ions of the peptide GPLTPLPV: AES-1 (128-135)*

No.	Seq.	*a*	*b*	*bo*	*d*	*y*	*yo*	No.
1	Gly	30.0	58.8	40.0	75.1	793.5	775.5	8
2	Pro	127.1	155.1	137.1	172.1	736.5	718.5	7
3	Leu	240.2	268.2	250.2	285.2	639.4	621.4	6
4	Thr	341.2	369.2	351.2	386.2	526.3	508.3	5
5	Pro	438.3	466.3	448.3	483.3	425.3	407.3	4
6	Leu	551.4	579.4	561.3	596.4	328.2	310.2	3
7	Pro	648.4	676.4	658.4	693.4	215.1	197.1	2
8	Val	747.5	775.5	757.5	792.5	118.1	100.1	1

*The values in the vertical columns indicate the residue masses of the peptides resulting from degradation of peptide G76, in the free amino and free acid form, starting from each terminus. The *bo*/yo ions differ from the corresponding *b* and *y* ions by the loss of one molecule of water (i.e. 58-(16+2)=40), while the *a* ions differ in mass from the *b* ions by the loss of a carbonyl group i.e. 58-(16+12)=30). The exact mass of peptide G76 (C38 H64 N8 O10) is 792.47. The exact mass of peptide G57 with the C terminal Pro-Val group reverted as compared to G76 is the same with G76.

TABLE II. Stabilization of HLA-A2 expression on T2 cells by AES peptides.

Code	Sequence	MCF-R[a] MA2.1	MCF-R[a] BB7.2
G76:	G P L T P L P V	1.76	1.03
G75:	L P L T P L P V	1.53	1.52
G77:	G P L T P L P V G L	1.51	0.96
G78:	L P L T P L P V G L	1.44	1.2
G60:	A L P L T P L P V	4.66	N.D.
G61:	A L A L P L T P L	3.06	2.80
G57:	G P L T P L V P	1.29	N.D.
G58:	G P L T P L V P G L	1.07	N.D.
G59:	P L T P L V P G L	1.58	N.D.

[a]Represents the mean channel fluorescence ratio (MCF-R), between the MCF corresponding to T2 cells incubated with any of the peptides and T2 (control) that have not been incubated with peptide (T2/NP). Each peptide was used at a concentration of 20 μg/ml.

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VACCINE IMPLICATIONS OF FOLATE BINDING PROTEIN, A NOVEL CYTOTOXIC T LYMPHOCYTE-RECOGNIZED ANTIGEN SYSTEM IN EPITHELIAL CANCERS

GEORGE E. PEOPLES,[1,7*] BRETT W. ANDERSON,[2] JAMES L. MURRAY,[3] ANDREZJ P. KUDELKA,[4] TIMOTHY J. EBERLEIN,[5] J. TAYLOR WHARTON,[2]
and CONSTANTIN G. IOANNIDES[2,6]

Departments of [1]Surgical Oncology,[2]Gynecologic Oncology, [3]Bioimmunotherapy, [4]Gynecologic Medical Oncology, and [6]Immunology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, [5]Department of Surgery, Washington University, St. Louis, MO, 63110,[7] Uniformed Services University of the Health Sciences, Bethesda, MD 20814.

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ABSTRACT The immune system can be efficiently stimulated and targeted to specific antigens expressed exclusively or preferentially by experimental cancers. The foremost limitations to extending this vaccine technology to the prevalent epithelial-derived cancers are (a) the lack of identification of tumor-associated antigens (TAA) recognized by cellular immunity and (b) the lack of antigen expressed on the majority of tumor cells during disease progression. To date, only HER2/neu (HER-2) has been shown to be the source of naturally occurring, MHC-restricted cytotoxic T lymphocyte (CTL)-recognized peptides in epithelial tumors. In this study, we demonstrate that the human high affinity folate binding protein (FBP) is a source of antigenic peptides recognized not only in ovarian cancer but also in breast cancer. Both immunodominant E39 (FBP, 191-198), and subdominant E41 (FBP, 245-253) epitopes are presented by HLA-A2 in these cancers. These peptides are efficient at amplifying the response of tumor-associated lymphocyte (TAL) populations in terms of lytic function, enhanced proliferation, and peptide-specific IFN-γ release. Furthermore, on a per cell basis TAL stimulated with the FBP peptides exhibit enhanced cytotoxicity not only against peptide-loaded targets but also against FBP-expressing epithelial tumors of different histologies. The broad distribution of FBP among >90% of ovarian and endometrial carcinomas, as well as 20-50% of breast, lung, colorectal, and renal cell carcinomas, along with pronounced differential expression in malignant tissues compared to the extremely limited expression in normal epithelium, suggests the exciting potential of a widely applicable FBP-based vaccine in epithelial cancers.

INTRODUCTION

Anti-cancer vaccines have taken many forms from whole tumor cells (WTC) to viral oncolysates (VO) to most recently antigen-specific, peptide-based vaccines. The majority of preclinical and clinical data has been generated in WTC and VO vaccine studies, and against this data, all other vaccines are compared (1-5). The theoretical advantage of these vaccines is that the actual cytotoxic T lymphocyte (CTL)-recognized antigens do not need to be known. The disadvantages, however, include safety issues of introducing tumor cells, activated oncogenes, and viruses into patients. Furthermore, many irrelevant proteins are also delivered, resulting in low levels of relevant antigen presentation. Genetic modification of WTC with cytokine genes or costimulatory genes has resulted in tumor regression in animal models, but so far has not been successful in humans (6-8).

Tumor associated antigens (TAA) may be partially purified or highly purified/ synthetic in nature. The pure peptide antigen (Ag) vaccines (or genes encoding for these Ag) should offer the best theoretical advantage over WTC and VO vaccines by delivering the specific immunogenic antigen(s) capable of inducing an efficient, specific tumor-protective immunity without the irrelevant antigens or need of autologous or allogeneic tumor material. The TAA vaccines can be easily and reproducibly manufactured, and delivered in high volumes safely.

To extend the advances in vaccine technology to the vast problem of epithelial cancers, CTL-recognized TAA must be identified in epithelial tumors. Such TAA should meet the criteria of high and stable expression in tumor cells of a large number of patients during disease progression to allow targeting. To date, only the protein product of the proto-oncogene, HER2, and the core peptide of MUC-1, have been shown to be a source of CTL-recognized peptides (9-12). The advantage of the HER-2 antigen system over those known for melanoma is that it is expressed in multiple epithelial-derived tumor histologies like breast, ovarian, pancreas, and non-

small cell lung carcinomas making a HER2 peptide-based vaccine potentially widely applicable (13-14). However, this protein is only overexpressed in 30% of breast and ovarian cancers and less in others (15).

Folate binding protein (FBP) is a membrane-associated glycoprotein originally found as a mAb-defined antigen in placenta and trophoblastic cells but rarely in other normal tissues (16-18). Of interest, this protein has been found in >90% of ovarian and endometrial carcinomas; in 20-50% of breast, colorectal, lung, and renal cell carcinomas; and in multiple other tumor types (19). When present in cancerous tissue, the level of expression is usually >20-fold normal tissue expression (18), and has been reported to be as high 80-90-fold in ovarian carcinomas (20). While FBP has been investigated extensively as a target of humoral immunity, it has only recently been proposed as a source of CTL-recognized peptides in ovarian cancer.[§] To further investigate the role of FBP peptides, we have studied whether these peptides are immunogenic in breast and ovarian cancer patients, and whether FBP peptides can amplify CTL-TAL with broad tumor-killing capabilities.

In this study, we demonstrate that two FBP-peptides are recognized not only by freshly cultured ovarian cancer-associated lymphocytes, (OvTAL) but also by breast cancer-associated lymphocytes (BrTAL). These peptides correspond to naturally processed antigens on intact tumor cells. Additionally, FBP peptides are capable of CTL stimulation *in vitro* resulting in proliferation, peptide-specific cytokine release, and enhanced cytotoxicity. FBP peptide-stimulated specific CTL are capable of lysing multiple tumors with different epithelial-derived histologies.

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MATERIALS AND METHODS

Tumor-Associated Lymphocyte Cultures. TAL were isolated from fresh collections of malignant ascites and pleural effusions from ovarian and breast cancer patients, respectively. These materials were obtained through the Department of Gynecologic Oncology at U. T. M. D. Anderson Cancer Center, Houston, TX, under the approval of the Institutional Review Board. Specimens were processed as previously described (21). Briefly, sterile samples of malignant ascites and pleural effusions were collected in heparinized containers and immediately transported to the laboratory. The cellular elements of these fluids were obtained by centrifugation and washed with serum-free RPMI-1640. Once resuspended, the lymphocytes and tumor cells were separated by centrifugation over discontinuous 75%/100% Ficoll-Histopaque (Sigma, St. Louis, MO) gradients. Freshly isolated TAL were suspended in RPMI-1640 containing 100 μg/ml L-glutamine (Gibco, Grand Island, NY) supplemented with 10% FCS (Sigma), 40 μg/ml gentamicin, and 50-100 IU/ml IL-2 (Cetus, Emeryville, CA), and cultured at 0.5-1.0 x 10^6 cells/ml in a humidified incubator at 37^0 C in 5% CO_2 and maintained at this concentration by the addition of media and IL-2 every 2-3 days depending on the growth kinetics.

Tumor Targets. The SKOV3 ovarian cancer cell line (ATCC, Rockville, MD) was transfected with the HLA-A2 expression vector RSV.5-neo with resulting high levels of HLA-A2 expression (SKOV3.A2) as described (22). The SKBr3 breast cancer cell line (ATCC) was similarly transfected with the HLA-A2 gene (by Drs M. Disis and M. Cheever, University of Washington, Seattle) but expresses moderate levels of HLA-A2 (SKBr3.A2) when compared to SKOV3.A2. Both lines were maintained in RPMI-1640 with 10% FCS and 250 μg/ml G418 (Sigma). SW480 is an established, well-characterized HLA-$A2^+$ colon cancer cell line (ATCC),

and PAN-1 is a HLA-A2$^+$ pancreatic cancer cell line (ATCC). SKBr3.A2, SKOV3.A2, and SW480 express HER2 with decreasing levels in this order. SKOV3.A2, SW480 and Panc-1 have been shown to express FBP (19,23).

Phenotype Analysis. The HLA-A2 status of the TAL lines and tumor lines was determined by indirect staining with anti-HLA-A2 mAbs, BB 7.2 and MA 2.1 (ATCC) followed by goat anti-mouse mAb conjugated with FITC (Becton Dickinson, Mountain View, CA) and analyzed on a Coulter Epics C Flow Cytometer (Coulter Electronics, Hileah, FL). HER2 expression was tested similarly utilizing the Ab2 mAb (Oncogene Science, Manhasset, NY). FBP expression was analyzed using the MOv18 mAb generously donated by Centocor (Malvern, PA).

Synthetic Peptides. The FBP sequence was interrogated for potential HLA-A2-binding nonamers utilizing the known binding motifs for this molecule (24). Five peptides were selected for synthesis based on the presence of leucine, isoleucine, or valine in the dominant anchor positions, P2 and P9, and their potential to form amphiphilic helices. These peptides and their binding affinities have been recently described.[§] Peptides were prepared by the Synthetic Antigen Laboratory of U. T. M. D. Anderson Cancer Center. Identity and purity of final materials were established by amino acid analysis and analytical RP-HPLC. All peptides utilized in this study were between 92-95% pure. Briefly, the symbols, position and sequence of the peptides utilized in this study are as follows: E37 (FBP, 25-33) RIAWARTEL; E38 (FBP, 112-120) NLGPWIQQV; E39 (FBP, 191-199) EIWTHSTKV; E40 (FBP, 247-255) SLALMLLWL; E41 (FBP, 245-253) LLSLALMLL. All of these peptides are low to moderate binders except E38 which is a high affinity binder to HLA-A2.

Cytotoxicity Assays. Cytotoxicity was determined by standard chromium-release assays as previously described (21). Briefly, tumor targets were labelled with 100-150 μCi of sodium chromate (^{51}Cr) (Amersham, Arlington Heights, IL) for 1.5 hr at 37°C, washed twice and plated at 2000-2500 cells/well in 100 μl in 96 well v-bottom plates (Costar, Cambridge, MA). Effectors were added at designated effector:target (E:T) ratios in 100 μl/well. After 5-20 hr of incubation, 100 μl of culture supernatant was collected, and ^{51}Cr release was measured on a gamma counter (Gamma 5500B, Beckman, Fullerton, CA). All determinations were done in triplicate. Results are expressed as percent specific lysis as determined by: (experimental mean cpm-spontaneous mean cpm)/(maximum mean cpm-spontaneous mean cpm) x 100.

Peptide-Pulsed Cytotoxicity Assays. For these experiments, the T2 line (generously donated by P. Creswell, Yale University) was utilized. T2 is a human T cell/B cell fusion product containing an antigen-processing defect in the TAP proteins such that HLA-A2 molecules are empty on the cell surface or contain relatively few bound peptides which can be effectively displaced by exogenous HLA-A2-binding peptides (26). The T2 cells were labelled with chromium as above, washed, and then incubated with peptide for 1.5 hr at 37°C prior to standard cytotoxicity assays. To increase the sensitivity of detection at low E:T ratios by uncloned effectors, 20 hr CTL assays were used in parallel with the 5 hr assays. T2 without peptide (T2/NP) was also used as a control. For cold target inhibition assays, unlabelled T2 were incubated with peptide for 1.5 hr then added to standard cytotoxicity assays with chromium-labelled tumor targets and effectors. The cold-hot target ratio was 15:1.

CTL Induction Experiments. Freshly cultured TAL were plated at 1 x 10^6 cells/ml in 24-well culture plates (Costar) in RPMI-1640/10% FCS without IL-2. T2 cells were irradiated

with 100 Gy (Cesium source), washed, and incubated with saturating concentrations of individual FBP peptides for 1.5 hr prior to being added to six parallel TAL cultures at a 10-15:1 responder: stimulator ratio. After 48 hr, 50 IU/ml of IL-2 was added. Media and IL-2 were then added every 2-3 days as needed. Parallel control cultures were established with T2 cells and maintained in the exact same manner except for the omission of FBP peptide (T2/NP). After one week in culture, cells were counted and the proliferation index calculated as a ratio of peptide-stimulated culture cell number to the control cultures stimulated with T2/NP. This approach was preferred over DNA synthesis as thymidine incorporation as it indicates the recovered live cells after stimulation. Cytotoxicity assays were performed as above at one week from the last stimulation.

Cytokine Release Assays. Peptide-stimulated parallel cultured TAL were replated at 1 x 10^6 cells/ml in 1 ml after a week and restimulated in the same fashion as described above. Supernatants from the parallel cultures were harvested at 24 and 48 hr prior to the addition of IL-2 and stored at -20^0C until analyzed. Supernatants were also harvested at 24 and 48 hr after the initial stimulation of the freshly cultured parallel TAL. Peptide-specific cytokine release from the TAL was measured in 50 µl for IFN-γ and IL-4 at the two time points utilizing ELISA kits (BioSource, Camarillo, CA) with a sensitivity of 4 pg/ml according to the manufacturer's instructions. Results are given as pg/ml produced by 1 x 10^6 cells.

RESULTS

Ovarian and Breast Cancer-Associated Lymphocytes Recognize FBP-Peptides. Four consecutive HLA-A2$^+$ malignant ascites specimens from ovarian cancer patients and two malignant pleural effusions from breast cancer patients were processed, and the TAL isolated and cultured in media containing IL-2 without restimulation with autologous tumor. Cytotoxicity assays utilizing peptide-loaded T2 cells as targets were performed with the TAL within 7-14 days of culture initiation in order to limit the *in vitro* expansion of irrelevant clones. The results in Figure 1*A* shows specific recognition by BRTAL-1 of FBP peptide E39 compared with another FBP peptide, E37, or control T2 cells which were not pulsed with peptide (T2/NP). The presence of CTL with this epitope specificity was confirmed by retesting the E39 recognition 8 days later, (Figure 1*B*) at higher E:T ratio (30:1) and higher Ag concentration (50 μg/ml). Since these results indicated that the FBP peptide specificity is present among the tumor associated CTL and stable within the first three weeks of culture, additional ovarian and breast TAL were screened. Figure 1*C* shows the pooled results of 18 experiments utilizing as effectors the six fresh ovarian and breast TAL cultures. For comparative purposes, individual assays were normalized with respect to background nonspecific lysis which ranged from 4-8%. E37, a low affinity binder, and E38, a high affinity binder, were not significantly recognized in these assays and, therefore, served as internal specificity controls. E39-pulsed T2 resulted in the highest cytotoxicity by TAL (Fig 1*C*), and was most consistently recognized by both ovarian and breast TAL. E41 was highly recognized by one breast TAL culture but not the other. This is consistent with the OvTAL recognition of E41.[§] The recognition of these peptides by freshly cultured, *in vitro* unstimulated TAL documents the presence of precursor CTL specific for these epitopes *in vivo* suggesting *in vivo* priming to these antigens. E39 (FBP, 191-198) appears to be immunodominant FBP epitope while the overlapping peptides E40 (FBP 247-255) and E41 (FBP

245-253) form subdominant epitopes since both induced less frequent and more variable CTL recognition compared with E39.

FBP-derived Peptides are Naturally Processed Antigens. To determine whether FBP-E39 reconstitute T cell epitopes that are presented on ovarian tumor cells, cold target inhibition assays were performed. T2 were pulsed with E39 (T2/39) and utilized to block the recognition of TAL populations for the ovarian cancer cell line, SKOV3.A2. T2/39, but not T2/37 or T2/NP, effectively inhibited the tumor lysis by OVTAL1 (Fig. 2*A,B*) and OVTAL4 (Fig. 2*C,D*) in 5 (Figure 2*B, D*) and 20 hr (Figure 2*A, C*) cytotoxicity assays. The inhibitory effects of T2/E39 increased over time, between 5-20 hr, suggesting that the recognition of the epitope formed by E39 was not transient or nonspecific. These experiments were repeated, and the results were confirmed (data not shown). These findings suggest that the CTL specific for the E39 epitope contribute significantly to the recognition of this ovarian cancer cell line. Furthermore, these data demonstrate that FBP-derived peptides are naturally processed antigens.

Induction of Proliferation and Specific IFN-γ Release by FBP Peptides E39 and E41. Short-term cultured TAL were split into parallel cultures and stimulated with irradiated T2 loaded with either E37, or E39, or E41, or T2/NP as a control. Since T2 are allogeneic to the responders, a certain level of proliferation and cytokine secretion to T2/NP was expected. T2/37 was utilized as an internal specificity peptide control. E41 was chosen over E40 as the subdominant epitope to study since it was highly recognized by some TAL. One week later, the cells in these cultures were counted and proliferation index determined as compared to the T2/E37 and T2/NP-stimulated cultures. Figure 3*A* shows the effect of E39 and E41 stimulation on OvTAL1 and OvTAL2 cell numbers. Both peptides induced enhanced proliferation over the

level observed with T2/NP in both populations; E39 was superior to E41 in OvTAL1 while E41 was better in OvTAL2 suggesting differences in precursor frequency and state of activation between the responders in these cultures.

E39 and E41 induced specific IFN-γ release in both peptide-stimulated and freshly cultured TAL populations. Both E39- and E41-stimulated OvTAL2 as well as freshly cultured OvTAL1 demonstrated peptide-specific IFN-γ release at 24 hr and the release increased at 48 hr. The amount of cytokine release was greater from the peptide-stimulated cultures (Figure 3*C*) compared with the freshly cultured TAL (Figure 3*B*). No IL-4 was produced in response to these peptides in either TAL culture (data not shown). These findings suggest that the FBP peptides have the ability to activate IFN-γ secretion which may be relevant for the cytokine-mediated effector pathway.

Enhanced Cytotoxicity of FBP Peptide-Stimulated TAL. One week after primary FBP peptide stimulation, the TAL cultures were evaluated for specific Ag recognition. One week after a single stimulation with T2/39, the TAL populations revealed an increase in lytic activity, on a per cell basis for E39, that ranged from negligible (10%) for OvTAL3 to 5-fold, (OvTAL-4) compared to parallel cultures stimulated with T2/NP (Fig. *4A*). Results with OvTAL2 are shown in Fig. 4*B*. These results indicate a requirement for the relevant peptide during stimulation in CTL expansion and expression of the lytic function. To better evaluate Ag and tumor specificity after one peptide stimulation, we focused on the weak responders OvTAL-1 and OvTAL-2. Expanded Ag recognition assays are shown for OvTAL2 and OvTAL1 (Fig. 4, B, C, D). OvTAL2 stimulated with the control peptide, E37, demonstrated nonspecific recognition for the peptide-loaded targets (Fig. 4*B*). OvTAL2 stimulated with E39 also showed weak specificity for E39 (25% over controls), while E41-stimulated OvTAL2 exhibited marginal

specificity to E41 (38% over control T2/E37). Of interest, the weakly enhanced E41 specificity produced also a modest 20% increase in SKOv3.A2 lysis compared to the other peptide-stimulated OvTAL2 cultures (Fig. 4*B*).

Importantly, OvTAL1, which proliferated better in response to E39 at primary stimulation showed high specificity for E39 after re-stimulation. This was detectable at an E:T ratio as low or 2.5:1 and confirmed in 20 h assays (Figure 4*C, D*). The parallel cultures re-stimulated with T2/NP, T2/37, and T2/41 revealed no Ag-specificity in either 5 h (Fig. 4*C*) or in 20 h assays (Fig. 4*D*). The E39-stimulated TAL elicited substantially better lysis of SKOV3.A2 than the cultures stimulated with any other peptide (also compared in Fig. 2*A*). These results demonstrate the immunogenicity of E39 (FBP, 191-198) as a naturally occurring T cell epitope on ovarian cancer for Ag-primed CTL, leading to effectors capable of effectively lysing a FBP-expressing tumor. These results indicate that, even under conditions of allogeneic stimulation which are expected to amplify an alloreactive response, re-stimulation of CTL with the self-peptide tumor Ag, E39, succeeded in amplifying a CTL response to the tumor after two stimulations.

Epithelial Tumor Lysis by FBP Peptide-Stimulated TAL. In separate experiments, OvTAL1 and OvTAL2 stimulated twice by FBP peptide-loaded T2 over 2 weeks were evaluated for recognition of multiple tumor targets at a low E:T ratio of 2.5:1. OvTAL2 stimulated with the control-FBP peptide, E37, demonstrated no recognition of the breast line SKBr3.A2 (HLA-2^{low}, HER2/neu^{high}, FBP^{+}) or the colon line SW480 (HLA-$A2^{high}$, HER2/neu^{low}, FBP^{+}), but did demonstrate some baseline recognition of the ovarian line SKOV3.A2 (HLA-$A2^{high}$, HER2/neu^{high}, FBP^{+}) (Figure 5*A*). OvTAL2 restimulated with E41 demonstrated a higher recognition of SKOv3.A2 compared to the E39-or E37-stimulated OvTAL2, but their ability to

lyse SKOV3.A2 was not enhanced (compare with the control Figure 4*B*). The low HLA-A2-expressing SKBr3.A2 was weakly recognized (Fig. 5*A*). In assessing the ability of these TAL to lyse FBP-expressing epithelial tumors of different histology, the E39-stimulated OvTAL2 was superior to E37- or E41- stimulated OvTAL2 in recognizing the SW480 colon line (Figure 5*A*) suggesting differences in epitope presentation by these tumors. The recognition of FBP-expressing epithelial tumors was confirmed by the OvTAL1 effectors stimulated with E39. These effectors lysed not only SKOV3.A2 and SW480 but also the pancreatic line, PAN-1 (HLA-A2^{high}, HER2/neu^{high}, FBP^{+}) but weakly the control tumor, SKBr3.A2 (Fig. *5B*). Of interest, the levels of SKOV3.A2 lysis observed with 10^4 effectors in this assay were similar with the levels observed with 8 X 10^4 unstimulated effectors (Fig. 2) of the same line, indicating that T2/E39 stimulation lead to an enrichment in E39 specific effectors. Both E39-stimulated, OvTAL1 and OvTAL2 cultures revealed substantial lysis of the low HER2-expressing, FBP^{+} colon line SW480. Since all these tumors express HER2, one might suggest that this antigen system is being recognized; although, one would expect less HER2 recognition of the lower HER2-expressing tumor SW480. This was not seen in these experiments. Therefore, as demonstrated in these studies, the broader expression of FBP relative to HER2 would make FBP the preferential target antigen system in epithelial cancers.

DISCUSSION

In this study, we found that six freshly cultured ovarian and breast TAL not previously subjected to antigen stimulation (anti-CD3, autologous tumor) *in vitro* recognize FBP peptides, particularly E41 (FBP, 245-253) and most consistently E39 (FBP, 191-199). Cold target inhibition studies demonstrate that E39 and E41 represent peptides that are naturally expressed epitopes on ovarian cancer cells. Together these data prove that: (a) FBP functions as a TAA recognized by CTL-mediated immunity in these cancers: (b) FBP peptide E39 is efficient in amplification of specific CTL.

E39 and E41 peptide-stimulated CTL were capable of lysing FBP-expressing tumors of different histologies. We selected three different tumor types for this study that have been shown by different groups using biochemical, immunohistochemical, and binding approaches to express FBP (22, 27). We found that E39 and E41-stimulated ovarian TAL not only could recognize allogeneic HLA-$A2^+$ ovarian cancer cells, but also lysed other FBP-expressing HLA-$A2^+$ epithelial tumor cells. We are currently developing novel approaches by isolating tumor cell membranes followed by immunoprecipitation with Mov18 mAb and Western blotting to quantitate FBP expression in a large panel of epithelial tumors and to correlate this expression with sensitivity to CTL lysis.

FBP peptides have the ability to activate both cytolysis and cytokine (IFN-γ)-secretion. The latter has been shown to be important in CTL induction and maturity. Furthermore, IFN-γ enhances HLA class I expression and endogenous antigen-processing by upregulating several key components including proteasomes and peptide transporters (26). IFN-γ also promotes the activation of Th1 responses while inhibiting Th2 cells. Additionally, IFN-γ influences the level of expression of some known antigens such as HER2 (27).

One of the most promising aspects of epithelial cancer vaccine development is that epithelial tumors share common CTL-recognized epitopes, (29) indicating that a TAA-specific vaccine may be widely applicable. Until now, only MUC-1 and HER2 have been identified as sources of antigenic peptides. SKOV3.A2, SW480, and PAN-1 were chosen for this study because they differ in their level of HER2 expression. If the HER2 antigen system alone was responsible for the common recognition of these tumors demonstrated in this report, the tumor with the lowest level of HER2/neu expression should have been the least sensitive to lysis by CTL. This was not the case. In fact, both E39-stimulated TAL populations efficiently lysed SW480 (HER-2^{lo}) better than SKBR3.A2. SKBR3 are among the highest expressors of HER-2. Since HER2/neu is only overexpressed in a minority of ovarian and breast cancers (15) these results further illustrate the clinical potential for targeting a widely expressed tumor antigen like FBP.

FBP was originally identified independently by three lines of investigation as: *(1)* the LK26 antigen identified with a mAb raised against the choriocarcinoma cell line, Lu-75(c) (16), *(2)* the antigen recognized by MOv18 and MOv19 mAbs raised against an ovarian carcinoma membrane preparation (29), and *(3)* the high affinity FBP characterized from placenta and KB carcinoma cell lines (17). The true extent of FBP expression is still unknown because the mAbs currently utilized require more complex techniques than simple immunofluorescence staining to detect FBP (18-20). Because of some intrinsic differences between the LK26, MOv18, and MOv19 mAbs, different levels of FBP expression on some tumors have been reported. There is however, general agreement that only low levels of FBP expression exist in some normal tissues such as choroid plexus, lung, thyroid, kidney, and sweat glands. High levels of FBP are preferentially expressed in a wide range of cancerous tissue (18-20). The reason for this differential expression is unknown but one hypothesis involves the upregulation of the folate

receptor to compensate for the loss of the alternative folate processing pathway involving the THF reductase gene which is frequently deleted in cancer cells (30). The highest levels of expression of FBP have been found in ovarian carcinomas with more than 90% of all ovarian carcinomas expressing elevated levels of this protein (19,20). The levels of overexpression have been shown to be >20 fold that of normal tissue routinely (18) and reported as high as 80-90 fold in one study (20). The extent of FBP expression in other tumors is probably underestimated as eluded to above; however, multiple tumor types have been shown to overexpress the LK26/FBP antigen including 20-50% of colorectal, breast, lung, and renal cell carcinomas as well as many other tumor types (19).

Both immunodominant (10) and subdominant (9) epitopes have been described for HER2 protein. This concept appears to hold for FBP also with E39 serving as an immunodominant peptide with the most consistent and highest level of recognition among consecutive TAL populations. E41 appears to be a subdominant epitope with high levels of recognition among some ovarian and breast TAL cultures but not others. These observations are important for understanding the T cell repertoire responding to a processed self-antigen. Identification of two CTL epitopes on FBP may proffer a selective advantage for its use for cancer vaccination studies since FBP is expressed in outbread populations. In other systems of immunologic disease under $CD8^+$ cell control, shifts in epitope dominance have been described recently and may be favorable or unfavorable predictors of disease outcome (31). It is important to understand how these epitopes interact since stimulation with some peptides have been shown to enhance the response to recognized peptides (32,33).

The question of how to deliver CTL epitope-based vaccines is still unanswered. Soluble peptide vaccines with a single peptide and immunoadjuvant are currently in clinical trial for both melanoma (35) and the E75, HER2 peptide, but response data are not yet available. Our results

show that in patients tested, E39 was able to significantly stimulate specific proliferation and IFN-γ secretion by CTL-TAL. The latter response requires higher levels of TCR engagement than activation of CTL lytic activity (36), suggesting that the affinity of TCR for E39- HLA-A2 complexes may be high. We are currently investigating the dominant and subdominant epitopes from both HER2/neu (E75, GP2, C85) and FBP (E39, E41) in parallel in order to *(a)* assess the CTL precursor frequency to these peptides in TIL and TAL, *(b)* determine the ability of each of these epitopes to induce primary CTL with tumor killing ability and *(c)* determine the potential synergism of these epitopes in optimal CTL induction.

In developing an immune response to cancer, the critical test of vaccination strategy is the ability of the antigen to activate primary responses by naïve/unprimed effectors. This requires significantly stronger Ag signaling than activation of memory responses, or re-stimulation of established effector CTL clones (i.e., from CTL-TAL) (35,36). Primary, *in vitro,* stimulation of T-cells from PBMC of healthy donors with autologous denditric cells pulsed with E39 resulted in induction of specific recognition of E39 in CTL assays but of lower levels of IFN-γ from the same donor compared with the HER-2 peptide E75. In contrast with E75, E39 specific CTL activity is seldomly detected in healthy donors, in the absence of peptide stimulation suggesting that E39-specific CD8+ CTL precursors may be infrequent or tolerized (B.W. Anderson and G.E. Peoples, manuscript in preparation). This raises the question of whether the weak stimulatory ability of E39 epitope delivered as soluble peptide for IFN-γ secretion reflects its low binding affinity to HLA-A2 or instability. These factors ultimately impact the feasibility of developing a vaccine by this and other cancer antigens, with the immunogen in the soluble peptide form. Ongoing studies are investigating the possibility of immune-gene therapy for these types of epitopes by *(a)* delivering the minimal CTL epitope together with the endoplasmic reticulum translocation sequence using vaccinia vectors to the APC, and *(b)* enhancing targeting of the

epitope precursor protein to the MHC-I processing and presentation pathway by identification of the normal degradation induction signals that allow presentation of both dominant and subdominant epitopes (Castilleja, Anderson, manuscript in preparation).

As effective delivery systems are developed, the key to a successful epithelial cancer vaccine still depends on the discovery of CTL-recognized TAA to be targeted with the vaccine. FBP serves as an endogenous source of CTL-recognized epitopes which are naturally expressed on a large number of epithelial tumors; therefore, FBP peptides may serve as the basis of a widely applicable epithelial cancer vaccine. Clinically, these findings would support the development of a polyspecific vaccine composed of dominant and subdominant peptides from either a single or multiple TAA.

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FIGURE LEGENDS

Figure 1. Freshly cultured ovarian and breast TAL recognize FBP peptides. **(A)** HLA-$A2^+$ BrTAL isolated from a pleural effusion, cultured in IL-2 without specific stimulation was tested for recognition of FBP peptides E37 and E39 **(A)** E37, E39 and E40 **(B).** The experiments shown in Figure 1*A*, and 1*B* were performed using the same culture as effectors at 8 days from each other. Peptides were used at 25 μg/ml in **(A)** and 50 μg/ml in **(B).** E:T ratios were 20:1 and 30:1, respectively. **(C)** OvTAL (n=4) and BrTAL (n=2) were tested in standard 5-h ^{51}Cr-release assays for recognition of the indicator T2 cells, loaded with five FBP peptides (E37-E41) or no peptide (T2/NP) as a negative control. The assays were performed in triplicate at an E:T ratio of 20:1 and repeated 2-4 times for each effector. The results are expressed as % specific lysis ± SEM. Pooled data with all six TAL populations in 18 independent assays demonstates E39 to be the most consistently recognized FBP peptide. E40 and E41 are recognized by some TAL but not others. The results are expressed as % specific lysis ± SEM.

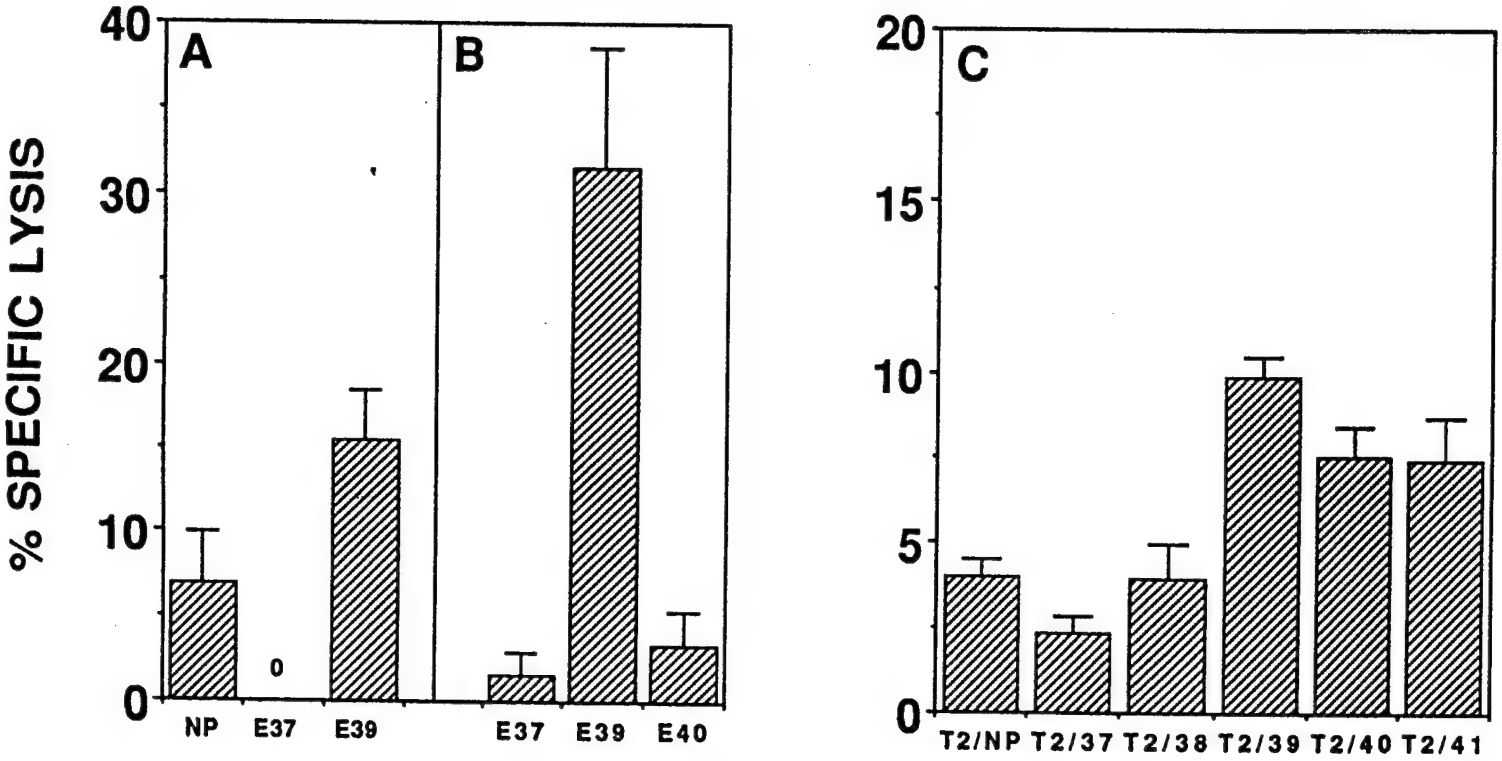
Figure 2. E39 (FBP, 191-199) reconstitutes an epitope corresponding to a naturally processed and presented antigen in ovarian cancer. Cold target inhibition assays were performed with OvTAL1 **(A, B)** and OvTAL4 **(C,D).** T2 loaded with E37 (negative control peptide), (●) E39 (■), or no peptide NP (O), were tested at a cold:hot ratio of 15:1 for inhibition of the recognition of the ovarian cancer cell line, SKOv3.A2 by OvTAL at E:T ratios of 10 and 20:1 in 5 h. **(B,D)** and 20 h **(A,C)** assays. Results are expressed as % specific lysis.

Figure 3. Stimulation with FBP peptides results in enhanced OvTAL proliferation and IFN-γ release. (A) OvTAL1 and OvTAL2 respond differently to FBP peptides. E39 ( ) and E41 ( ). OvTAL were cultured in parallel, and stimulated with irradiated T2 loaded with E37

( ), E39 ( ), E41, or no peptide (NP) ( ). Cell counts were performed after one week. Results are expressed as proliferation index, calculated by individually comparing the E39 and E41-stimulated cultures to the NP-stimulated parallel controls. (B) OvTAL1 and OvTAL2 demonstrate peptide-specific IFN-γ release. Parallel cultures of OvTAL-1 or OvTAL2 stimulated with T2 loaded with E39, E41, and NP were replated at 1 x 10^6 cells/ml in 1 ml and restimulated with T2 and the corresponding peptides E39 ( ), E41 ( ), E37 ( ) or T2/NP ( ) IFN-γ and IL-4 were measured 24 and 48 hours later. Results shown for IFN-γ are expressed as pg IFN-γ /ml as measured by ELISA from 50 μl culture supernatant. This result is representative of replicated experiments.

Figure 4. Enhanced cytotoxicity after FBP peptide stimulation. (A) Three OvTAL populations were stimulated in parallel with irradiated T2 loaded with E39 ( ) or T2/NP ( ). Recognition of E39 was assessed in standard CTL assays after one week at an E:T of 10:1. Results are expressed as % specific lysis ± SEM. (B) OvTAL2 was split, cultured in parallel, and stimulated with irradiated T2 loaded with E37 ( ), E39 ( ), or E41 ( ). After one week, these cultures were tested at an E:T=10:1 for recognition of these same peptide-loaded T2 targets in 5 hr CTL assays and of the tumor target, SKOv3.A2, in 20 hr assays. Results are expressed as % specific lysis ± SEM. ND=not done. (C) OvTAL1 was similarly stimulated as OvTAL2 in parallel with irradiated T2 loaded with E37, E39, E41, or NP twice at weekly intervals. The resulting cultures were tested in standard 5 h (C) and 20 h (D) cytotoxicity assays for recognition of peptide-loaded T2 and SKOv3.A2 at a lower E:T ratio of 2.5:1. Results are expressed as % specific lysis ± SEM. ND=not done.

Figure 5. FBP peptide stimulated ovarian TAL recognize FBP-expressing tumors of different histologies. (A) OvTAL2 stimulated twice at weekly intervals with irradiated T2 loaded with E37, E39, or E41 were tested for recognition of the low HLA-A2-expressing breast cancer line SKBr3.A2 (HLA-2^{low}, HER2/neu^{high}, FBP^{+}), ( ) the ovarian cancer line SKOv3.A2 (HLA-A2^{high}, HER2/neu^{high}, FBP^{+}), ( ) and the colon cancer line SW480 (HLA-A2^{high}, HER2/neu^{low}, FBP^{+}) ( ) in standard 20 hr cytotoxicity assays at an E:T ratio of 2.5:1. Results are expressed as % specific lysis $\pm$ SEM. (B) OvTAL1 was stimulated twice at weekly intervals with irradiated T2 loaded with E39 and tested for recognition for the same tumors above and also the pancreatic cancer cell line, PAN-1 (HLA-A2^{high}, HER2/neu^{high}, FBP^{+}) in standard 20 hr cytotoxicity assays at an E:T ratio of 2.5:1. Results are expressed as % specific lysis $\pm$ SEM.


% SPECIFIC LYSIS
40
30
20
10
0
A
B
0
NP
E37
E39
E37
E39
E40
20
15
10
5
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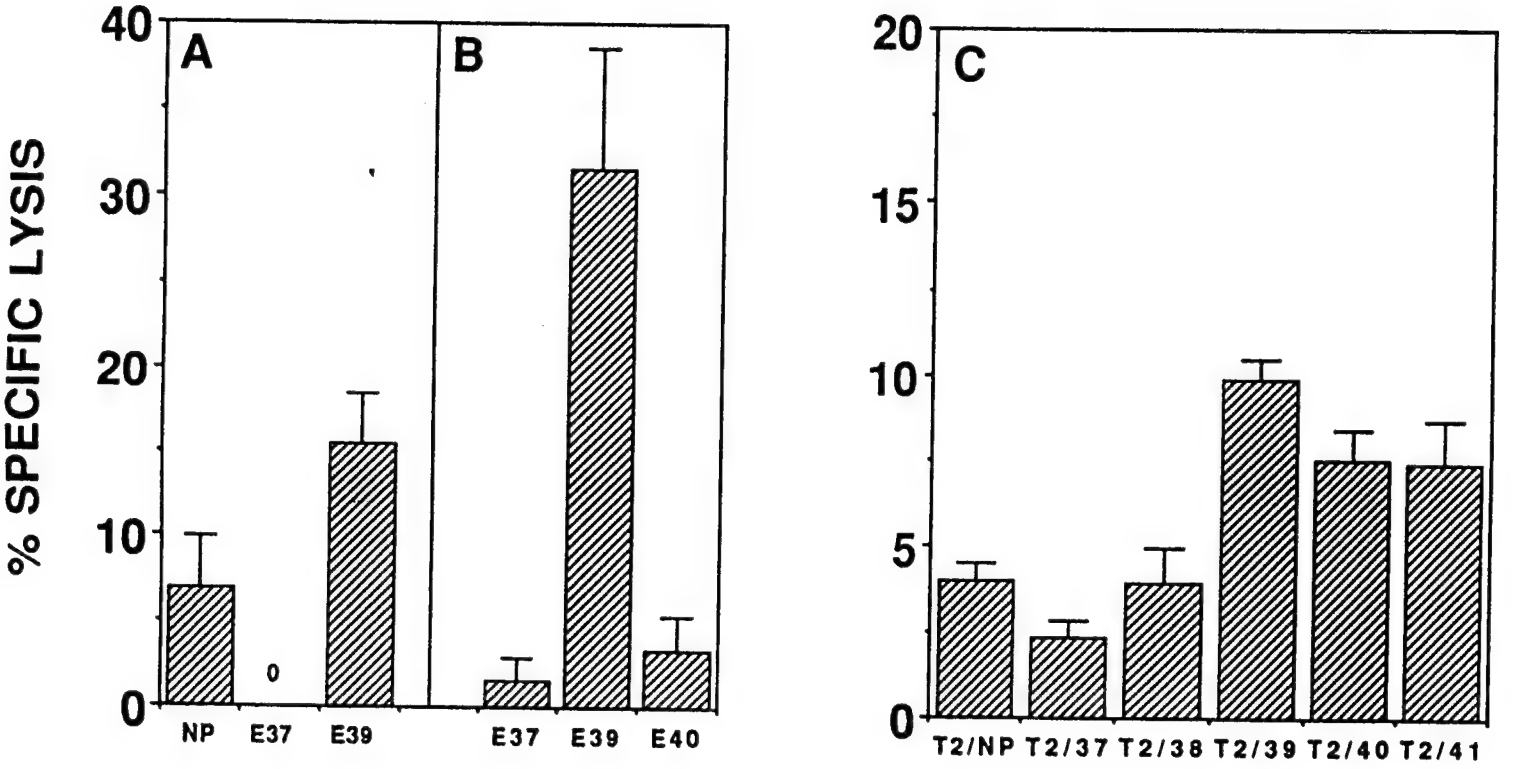
C
T2/NP
T2/37
T2/38
T2/39
T2/40
T2/41

Figure 1


% SPECIFIC LYSIS
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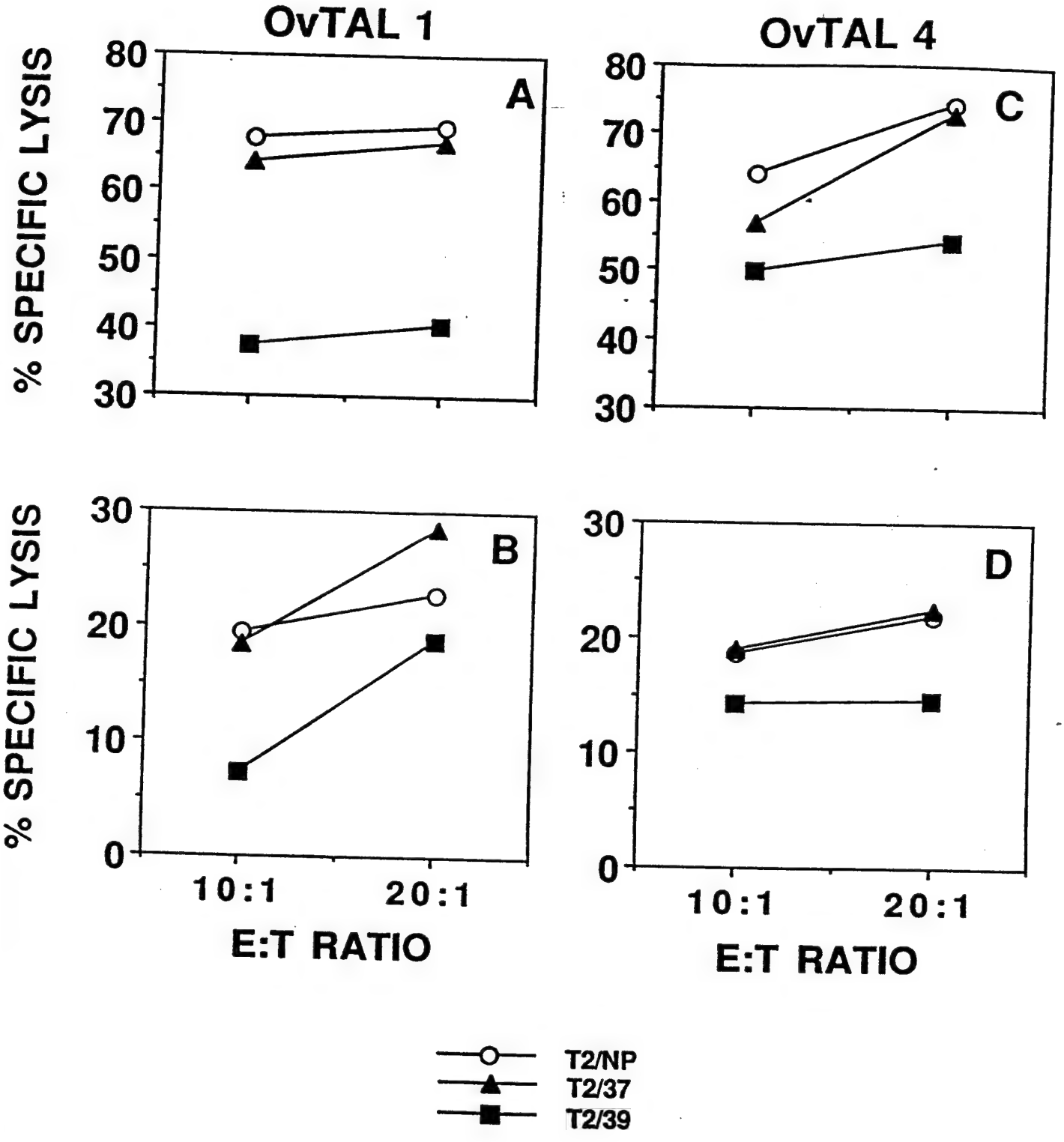
T2/40
T2/41

Figure 1


OvTAL 1
OvTAL 4
% SPECIFIC LYSIS
% SPECIFIC LYSIS
80
70
60
50
40
30
A
C
30
20
10
0
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D
10:1
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E:T RATIO
T2/NP
T2/37
T2/39

Figure 2

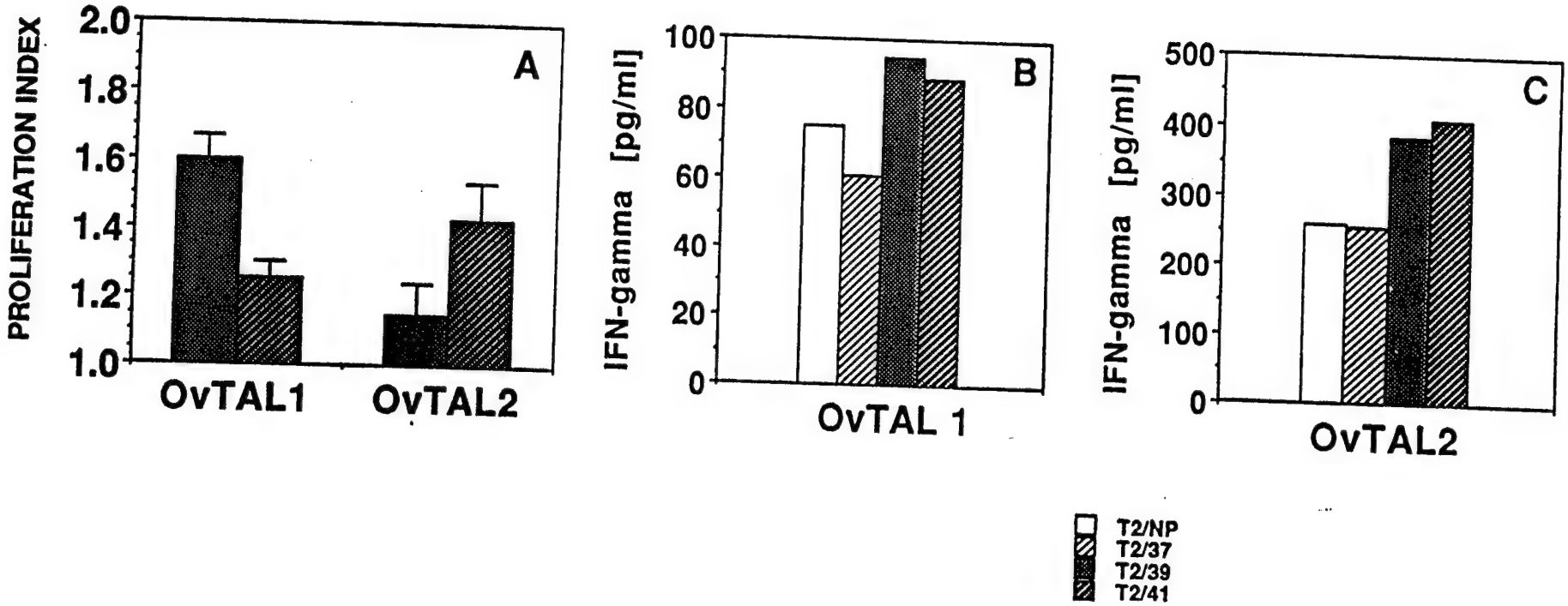

PROLIFERATION INDEX
2.0
1.8
1.6
1.4
1.2
1.0
A
OvTAL1
OvTAL2
IFN-gamma [pg/ml]
100
80
60
40
20
0
B
OvTAL 1
IFN-gamma [pg/ml]
500
400
300
200
100
0
C
OvTAL2
T2/NP
T2/37
T2/39
T2/41

Figure 3

FIG 4

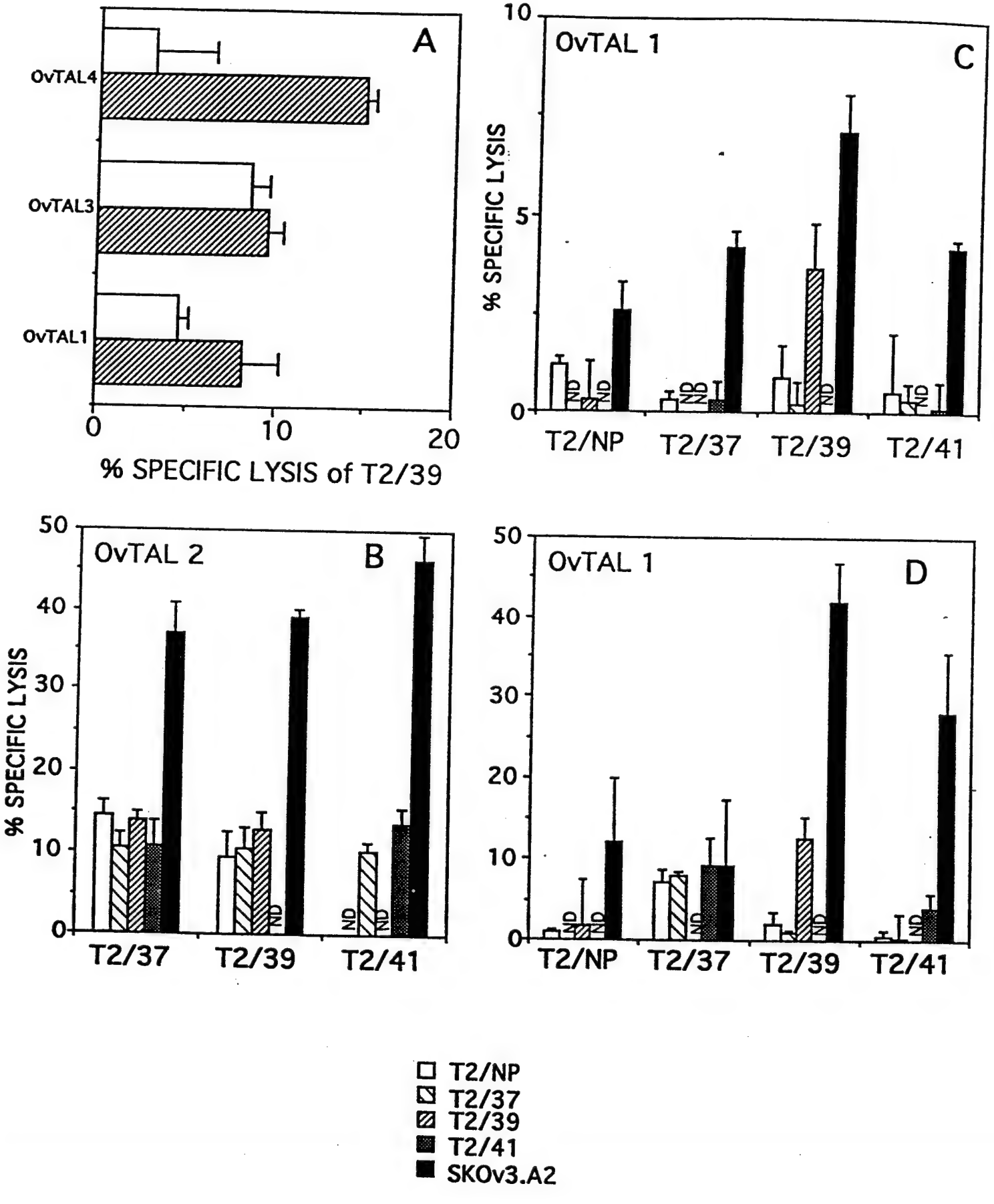
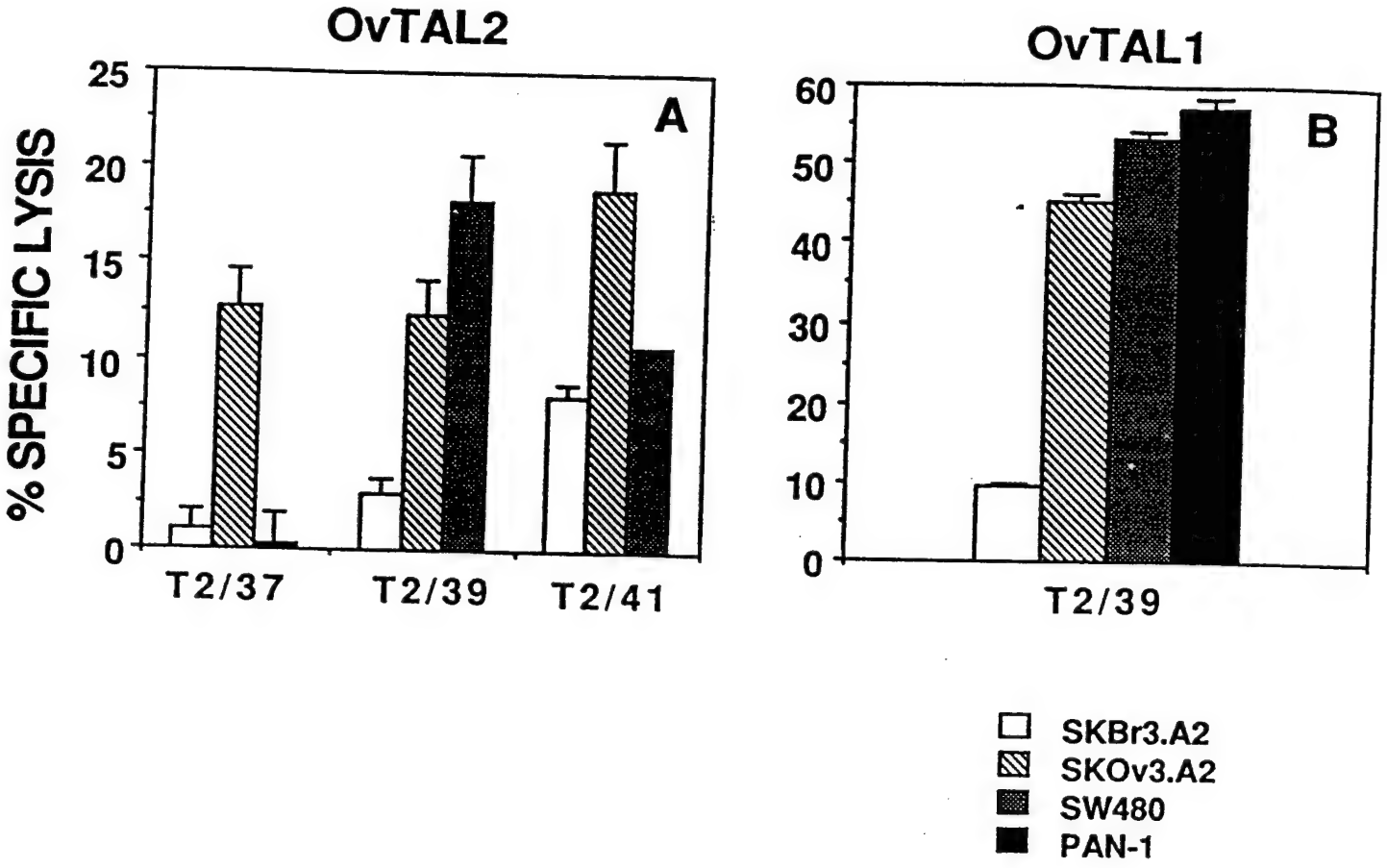

A
OvTAL4
OvTAL3
OvTAL1
0
10
20
% SPECIFIC LYSIS of T2/39
C
OvTAL 1
10
5
0
% SPECIFIC LYSIS
ND
T2/NP
T2/37
T2/39
T2/41
B
OvTAL 2
50
40
30
20
10
0
% SPECIFIC LYSIS
ND
T2/37
T2/39
T2/41
D
OvTAL 1
50
40
30
20
10
0
ND
T2/NP
T2/37
T2/39
T2/41
T2/NP
T2/37
T2/39
T2/41
SKOv3.A2

FIG 5


OvTAL2
% SPECIFIC LYSIS
25
20
15
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5
0
A
T2/37
T2/39
T2/41
OvTAL1
60
50
40
30
20
10
0
B
T2/39
SKBr3.A2
SKOv3.A2
SW480
PAN-1

OVARIAN CANCER-ASSOCIATED LYMPHOCYTES RECOGNIZE FOLATE BINDING PROTEIN (FBP) PEPTIDES

George E. Peoples, MD[1,5*] Brett W. Anderson, BS[2] Bryan Fisk, MS[2]
Andrzej P. Kudelka, MD[3] J. Taylor Wharton, MD[2]
and Constantin G. Ioannides, PhD[2,4]

Departments of [1]Surgical Oncology,[2]Gynecologic Oncology,
[3]Gynecologic Medical Oncology, and [4]Immunology
U. T. M. D. Anderson Cancer Center
1515 Holcombe Boulevard, Box 106
Houston, TX 77030
Phone=(713)792-8825
Fax=(713)792-0722

[5]Department of Surgery
Uniformed Services University of the Health Sciences
Bethesda, MD 20814

*To whom correspondence should be addressed.

Key words: CTL, peptide, vaccine, folate binding protein

Running Title: CTL-Recognized FBP Peptides

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ABSTRACT

BACKGROUND: Tumor-associated lymphocytes (TAL) isolated from ovarian cancer patients contain cytotoxic T lymphocytes (CTL) capable of recognizing specific HLA/peptide complexes on tumor cells leading to tumor cell lysis. Currently, HER2/neu, over-expressed in only 30% of breast and ovarian cancers, is the only known source of CTL-recognized peptides in epithelial cancers. Therefore, we have investigated peptides derived from folate binding protein (FBP) which is over-expressed in >90% of ovarian cancers and in the majority of other epithelial tumors.

METHODS: TAL were isolated from the malignant ascites of 4 consecutive HLA-A2$^+$ ovarian cancer patients and incubated in IL-2. Initial chromium-release assays were performed within 1 week. T2 cells, incubated with peptide, were used to reconstitute T cell epitopes. The FBP sequence was interrogated for HLA-A2 binding peptides, and 5 were synthesized (E37-41).

RESULTS: Freshly cultured, unstimulated ovarian TAL recognize peptides derived from FBP. These peptides are presented in the context of HLA-A2, and are specifically recognized in a HLA class I-restricted fashion. TAL recognition of these reconstituted T cell epitopes is concentration-dependent. Furthermore, the FBP peptides are shown to be naturally processed and presented antigens by cold target inhibition studies.

CONCLUSIONS: FBP peptides are recognized by freshly isolated TAL from ovarian cancer patients suggesting in vivo expression and sensitization. Since FBP is over-expressed 20-fold in most adenocarcinomas, these peptides may be utilized in a widely applicable peptide-based vaccine for epithelial tumors.

INTRODUCTION

A specific anti-cancer immune response has been well-established in melanoma and to a lesser degree in epithelial tumors. However, in ovarian, breast, lung, and pancreas cancers, tumor-specific cytotoxic T lymphocytes (CTL) have been isolated from tumors suggesting a host response (1-4). The most intensely studied epithelial tumor by far is ovarian cancer (5-9). This tumor has provided an invaluable model for the study of the specifics and similarities of the immune response to epithelial cancers as compared to melanoma. Ovarian cancer, which is the fourth leading cause of cancer death among American women (10), offers some unique advantages as a tumor model for immunologic research. This disease often presents in advanced stages with bulky disease and malignant ascites, and the primary treatment involves a staging laparotomy with tumor debulking resulting in large volumes of solid and ascitic tumor for laboratory use. Tumor-reactive CTL are readily and reproducibly isolated from both the solid tumors and ascites, and the latter can be recollected multiple times without surgery (1, 5-9). The motivation for immunologic alternative therapies is high in this disease since no effective treatments currently exist for women who fail primary platinum or taxol-based chemotherapy (10).

The classic interaction between the T cell receptor (TCR) on the CTL and the HLA/peptide complex on the tumor cell has been verified in the ovarian model, and like in melanoma, HLA-A2, which is expressed in 50% of Caucasions, has been confirmed as a restriction element (11). However, the most promising aspect of the ovarian cancer research to date, has been the discovery that common CTL-recognized, tumor-asscociated antigens (TAA) are

expressed not only on various ovarian cancers, but also on multiple other epithelial tumors. For example, we have shown that ovarian cancer-specific CTL also recognize common determinants on HLA-matched colon and pancreas cancers (12). Likewise, in separate studies, we have found that tumor-specific CTL isolated from HLA-matched ovarian and breast cancer patients are cross-reactive for tumor recognition (13).

In searching for these common TAA, we have also demonstrated the concept of shared TAA among various epithelial tumors by acid elution studies. Using an ovarian cancer cell line (14) and freshly isolated tumor cells (15), we have eluted the peptide antigens out of the HLA molecules, fractionated them, and reconstituted these epitopes on the HLA-A2$^+$ antigen processing defective mutant, T2 (16). We have shown that ovarian, breast, and non-small cell lung cancer-specific CTL recognize the same antigenic fractions further proving that common TAA exists among epithelial-derived tumors (14).

The identification of these TAA has progressed slowly despite the rapid developments in vaccine research in melanoma; however, one such antigen system has been found and confirmed by different groups. HER2/neu is a proto-oncogene, and its protein product has been shown to be the source of multiple peptides that are recognized by ovarian, breast, pancreas, and lung-cancer specific CTL (3, 4, 13, 17, 18). Fortunately, HER2/neu expression has been demonstrated in multiple epithelial-derived tumors (19, 20). However, since this is a normal, non-mutated protein, its usefulness as an immunologic target is dependent on its level of overexpression, and HER2/neu is only overexpressed in 30% of ovarian and breast cancers (21).

Folate binding protein (FBP), also known as LK26 trophoblast antigen (22) and GP38 (23), is a membrane-associated glycoprotein recognized by the monoclonal antibodies (mAb), LK26, MOv18, and MOv19 and found to be overexpressed in the vast majority of ovarian cancers (22, 24). The level of expression has been found to be >20-fold higher in malignant cells compared to normal cells (25), and in one study reported to be 80-90 fold higher (24). FBP has been the focus of many studies utilizing mAbs (26, 27), folate-conjugates (28, 29), and antifolates (30), but has not yet been investigated as a potential source of CTL-recognized peptides for use in anti-cancer vaccines.

In this study, we demonstrate that fresh TAL from consecutive ovarian cancer patients recognize FBP peptides in a HLA-A2-restricted fashion, and that these peptides are naturally processed antigens.

MATERIALS AND METHODS

Tumor-Associated Lymphocyte (TAL) Cultures - TAL were isolated from fresh collections of malignant ascites obtained through the Department of Gynecologic Oncology at U. T. M. D. Anderson Cancer Center, Houston, TX, under the approval of the Institutional Review Board. Specimens were processed as previously described (5). Briefly, malignant ascites was collected sterilely in heparinized containers and immediately transported to the laboratory. The cellular elements of the ascites was obtained by centrifugation and washed with serum-free RPMI-1640. Once resuspended, the lymphocytes and tumor cells were separated by centrifugation over discontinuous 75%/100% Ficoll-Histopaque (Sigma, St. Louis, MO) gradients. Freshly isolated TAL were suspended in RPMI-1640 containing 100 µg/ml L-glutamine (Gibco, Grand Island, NY) supplemented with 10% FCS (Sigma), 40 µg/ml gentamicin, and 50-100 IU/ml IL-2 (Cetus, Emeryville, CA). T cells were cultured at 0.5-1.0 x 10^6 cells/ml and placed in a humidified incubator at 37^0 C in 5% CO_2 and maintained at this concentration with the addition of media as needed and IL-2 every 2-3 days depending on the growth kinetics. Consecutive specimens were processed and cultured.

Tumor Targets- The SKOv3 ovarian cancer cell line (ATCC, Rockville, MD) was transfected with the HLA-A2 expression vector RSV.5-neo with resulting high levels of HLA-A2 expression as previously described (31). This cell line is maintained in RPMI-1640 with 10% FCS and 250 µg/ml G418 (Sigma). Fresh frozen tumor was collected from the malignant ascites after ficoll separation and frozen in aliquots in liquid nitrogen until used.

Phenotype Analysis- The HLA-A2 status of these TAL lines was determined by indirect staining with anti-HLA-A2 mAbs, BB7.2 and MA2.1 (ATCC) 50 µl of 1:50 dilution of culture supernatant at 4°C for 30 min followed by a 30 min incubation with goat anti-mouse mAb conjugated with FITC (Becton Dickinson, Mountain View, CA) and analyzed on a Coulter Epics C Cytometer (Coulter Electronics, Hileah, FL).

Synthetic Peptides- Peptides were synthesized in the Synthetic Antigen Laboratory of U. T. M. D. Anderson Cancer Center utilizing solid phase techniques on an Applied Biosystems 430 peptide synthesizer (Foster City, CA). Crude products were dissolved and injected onto C-18 4.6 mm I.D. reverse phase HPLC columns (Rainin) and eluted with linear TFA-acetonitrile gradients. Identity and purity of final materials were established by amino acid analysis and analytical RP-HPLC. All peptides utilized in this study were between 92-95% pure. All peptides were derived from the FBP sequence and contained I/L/V at the dominant anchor sites, P2 and P9, necessary for HLA-A2 binding.

HLA-A2 Stabilization Assays- Indirect assessment of peptide binding was perfromed by HLA-A2 stabilization assays as previously described (32). Briefly, T2 cells were pulsed with saturating quantities (100 µg/ml) of each peptide overnight. The cells were then washed and FACS analysis performed as above with BB7.2 and confirmed with MA2.1. HLA-A2 expression was then quanitated as mean channel fluorescence and compared to the expression level on non-peptide-loaded T2. Stabilization is expressed as a ratio of the HLA-A2 expression of peptide-loaded to unloaded T2.

Cytotoxicity Assays- Cytotoxicity was determined by standard chromium release assays as previously described (5). Briefly, targets were labelled with 100-150 μCi of sodium chromate (Amersham, Arlington Heights, IL) for 1.5 hr at 37°C then washed twice and plated at 2000-2500 cells/well in 100 μl in 96 well round bottom plates (Costar, Cambridge, MA). Effectors were added at designated effector:target (E:T) ratios in 100 μl/well. After 5-20 hr of incubation, 100 μl of culture supernatant was collected, and radionuclide release was measured on a gamma counter (Gamma 5500B, Beckman, Fullerton, CA). All determinants were done in triplicate. Results are expressed as percent specific lysis as determined by: (experimental mean cpm-spontaneous mean cpm)/(maximum mean cpm-spontaneous mean cpm) x 100.

Peptide-Pulsed Cytotoxicity Assays- For these experiments, the T2 cell line (generously donated by P. Creswell) was utilized which is a human T cell/B cell fusion product containing an antigen-processing defect in the TAP proteins such that HLA-A2 molecules are empty on the cell surface or contain relatively few bound peptides which can be effectively displaced by exogenous HLA-A2-binding peptides (16). The T2 cells were labelled with chromium as above, washed, and then incubated with peptide for 1.5 hr at 37°C prior to standard cytotoxicity assays.

mAb-Blocking Assays- Prior to standard cytotoxicity assays, peptide-pulsed T2 was incubated with anti-HLA-A2 mAb, BB7.2 (50 μl of 1:50 dilution of culture supernatant/well) or anti-HLA-A,B,C mAb, W6/32 (ATCC) (5 μl/well) for 30 min at 37°C prior to adding the effectors.

Cold Target Inhibition Assays- Unlabelled T2 was incubated with peptide for 1.5 hr then added to standard cytotoxicity assays with chromium-labelled tumor targets and effectors. The cold:hot target ratio was 15:1. T2 without peptide was also used as a control.

RESULTS

Folate Binding Protein-Derived Peptides- The FBP sequence was interrogated for potential HLA-A2-binding nonamers utilizing the known binding motifs for this molecule (8). Five peptides were selected for synthesis based on the presence of leucine, isoleucine, or valine in the dominat anchor positions, P2 and P9, and and the potential of these peptides to form amphiphilic structures (8). Peptides were selected with a wide range of predicted binding affinity. An indirect analysis of HLA-A2 binding was performed with HLA-A2 stabilization assays which are based on the concept that peptide affinity is directly proportional to enhanced HLA-A2 expression since peptides stabilize the HLA molecules on the cell surface of T2. Therefore, a peptide with high affinity will cause more HLA stabilization and higher expression. The peptide sequences, their positions, and their relative binding affinities are given in **Table I.** The HLA-A2 expression is given as a ratio of peptide induced expression over the expression on unloaded T2. Four of the peptides are relatively low-affinity binders while E38 is a high affinity binder.

FBP Peptide Recognition by Ovarian Cancer-Associated Lymphocytes- Four consecutive ovarian malignant ascites specimens, which were subsequently found to be HLA-A2$^+$, were processed and the TAL isolated and cultured in IL-2. Standard cytotoxicity assays were performed with the TAL populations within a week of culture initiation in order to limit in vitro artifact. **Fig. 1** shows the results of OvTAL1 and OvTAL4 at multiple E:T ratios against all five of the FBP-derived peptides and unloaded T2 as a control. E39-pulsed T2 resulted in the best cytotoxicity with both of these effector populations. The recognition of this peptide by fresh, unmanipulated TAL suggests that these effectors have

been previously exposed or primed to this epitope in vivo. To confirm these findings, all four TAL populations were tested against all five peptides in replicated assays performed in triplicate. These results are presented in **Fig. 2**. Several patterns of recognition emerged from these assays which are indicative of the different T cell repertoires present in the TAL populations. E37, a low affinity binder, and E38, a high affinity binder, were not significantly recognized in these assays and, therefore, served as negative peptide controls. E39 loaded T2 was the most consistently recognized target being lysed by 3/4 cultures and significantly overall when the data was pooled and compared to unloaded T2 or E37 and E38-pulsed T2 ($p<0.05$). E39 appears to be the immunodominant FBP-derived peptide while E40 and E41 may serve as subdominant peptides since each reconstituted T cell epitopes with variable recognition.

The Specificity of TAL Recognition of the FBP-derived Peptide, E39- To confirm the specific recognition of the HLA-A2/E39 peptide complex on the T2 cells by CTL, inhibition assays were performed by initially adding the anti-HLA-A2 mAb, BB7.2 to standard cytotoxicity assays. **Fig. 3A** demonstrates the successful inhibition of OvTAL2 lysis of T2/39 at multiple E:T ratios by blocking HLA-A2 in 5 hr assays. These data were confirmed with two TAL populations in 20 hr ^{51}Cr-release assays to enhance the sensitivity of the method as presented in **Fig. 3B**. In the latter set of experiments, anti-HLA class I mAb, W6/32 was also utilized to confirm HLA class I presentation of the peptide to CTL since the W6/32 mAb is a more effective blocker of cytotoxicity than BB7.2. These assays were repeated and similar results obtained.

Peptide Concentration-dependent TAL Recognition of FBP-derived Peptide, E39- To better understand the kinetics of CTL recognition of the E39 peptide, T2 cells were split into five parallel cultures and each pulsed with a different concentration of the peptide from 2-100 μg/ml for 1.5 hr prior to standard cytotoxicity assays with the same effector (OvTAL1) at a constant E:T ratio (**Fig. 4**). The optimal concentration was found to be 50 μg/ml with this low-affinity binding peptide, and half-maximal lysis occurred at the 2-5 μg/ml range. This is consistent with the range observed with HER2/neu peptides and most melanoma antigen peptides (13, 18, 33). Cytotoxicity dropped considerably at higher concentrations which has been a consistent finding in other peptide studies.

FBP-derived Peptides are Naturally Processed Antigens- In order to determine if FBP-derived peptides reconstitute T cell epitopes that are naturally processed and presented on ovarian tumor cells, cold target inhibition assays were performed. Cold T2 were pulsed with E39 and then utilized to block the cytotoxicity of TAL populations for the ovarian cancer cell line, SKOv3.A2. This cell line has been transfected with HLA-A2 in our laboratory and described previously (31). **Fig. 5** shows that T2/39 effectively inhibited 20-40% of the tumor lysis by OvTAL1 and OvTAL4 in multiple assays ($p<0.05$). These findings suggest that the CTL specific for this epitope contribute significantly to the recognition of this ovarian cancer cell line. Furthermore, this data demonstrates that FBP-derived peptides are naturally processed and presented antigens on intact ovarian tumor cells.

DISCUSSION

This study demonstrates that FBP is a source of antigenic peptides that induce an endogenous immune response as shown by the ability of freshly isolated and unmanipulated TAL to recognize several of these peptides, particularly E41 and most consistently E39. These peptides were recognized in a HLA-restricted fashion, and the cytotoxicity was concentration dependent. Furthermore, E39 was shown indirectly to be a naturally processed and expressed antigen on the ovarian cancer cell line, SKOv3.A2, since peptide loaded T2 could significantly inhibit CTL killing of this cancer line. Together this data strongly suggests that FBP is an endogenous TAA and is the source of antigenic peptides recognized by TAL in ovarian cancer.

Ovarian cancer has served as an extremely important model for the study of the immune response to epithelial cancer as demonstrated by the work of multiple groups (5-9). Most importantly, the findings have been extended to other less studied and technically more challenging epithelial tumor models. Furthermore, many of the findings in melanoma have been confirmed for epithelial cancers utilizing this model. Namely, we now know that an endogenous cellular immune response does exist in a variety of epithelial cancers, and that this response involves the specific recognition of antigenic peptides presented by HLA molecules, specifically HLA-A2, to specific TCR on tumor-infiltrating or tumor-associated CTL (7, 9, 11). Unlike melanoma, the investigation into the identity of these peptides has so far resulted in only one confirmed TAA as defined by recognition by cellular immunity. Only the protein product of the oncogene HER2/neu has been shown to serve as a source of endogenously recognized antigenic peptides (13, 18). Unfortunately,

this protein is only overexpressed in 30% of all ovarian and breast cancers (21). The advancements in anti-cancer vaccine research has been swift in melanoma and has been fueled by the ready supply of multiple commonly-expressed TAA (33). For further development of potentially widely applicable epithelial cancer vaccines, more CTL-recognized TAA must be found for epithelial tumors.

FBP was originally discovered from three independent lines of investigation. The LK26 antigen was indentified with a mAb raised against the choriocarcinoma cell line, Lu-75(c) by Rettig, et al (34). Initially, this antigen was found to be expressed in normal as well as malignant trophoblastic cells and eventually in ovarian carcinomas (22). The MOv18 and MOv19 mAbs were raised against an ovarian carcinoma cell membrane preparation and initially found to react with a cell surface glycoprotein with a molecular weight of 38 kDa (23). This protein was cloned and sequenced and found to be a high affinity FBP (35). Likewise, the latter protein was also characterized from placenta and KB carcinoma cell lines (36). The LK26 antigen was eventually found to be closely related or identical to the MOv18/MOv19 antigen (22).

The distribution of FBP expression is extremely interesting and relevant for immunotherapy. This protein is expressed in some normal specialized epithelium such as choroid plexus, lung, thyroid, kidney, and sweat glands but at very low levels (25). The highest levels of expression of FBP have been found in ovarian carcinomas, and in several independent studies, more than 90% of all ovarian carcinomas tested expressed elevated levels of this protein (22, 24). The levels of overexpression have been shown to be >20 fold that of normal tissue (25) and reported as high as 80-90 fold in one study (24). In

addition, multiple tumor types have been shown to overexpress the LK26/FBP antigen including 10/11 endometrial, 6/27 colorectal, 11/53 breast, 6/18 lung, 9/18 renal cell, 3/3 lung carcinoids, and 4/4 brain metastases from breast cancer. Mesotheliomas, lymphomas, sarcomas, and neuroectodermal tumors were either negative or rarely positive for FBP expression (22).

The fact that this TAA is so widely and differentially expressed among multiple epithelial tumor types makes it an ideal target for immunotherapy. Multilple attempts have been made to target therapeutic strategies toward FBP including folate conjugates and antifolates (28-30). Several studies have involved immunoconjugates or bi-specific mAbs similar to the studies targeting CA-125 (26, 27). The use of mAbs against this and other similar cell surface antigens has been limited as many of these mAbs have been raised against membrane extracts and may have less efficient recognition against the endogenously expressed conformational protein. Also, FBP is shed and much of the available mAb is bound to circulating antigen. Finally, much of the tumor is often inaccessible to circulating mAbs, and they are rapidly cleared by the host. For these and other reasons, developing mAb-mediated therapies targeting FBP has been challenging. However, the fact remains that most ovarian carcinomas drastically overexpress FBP, and this antigen is endogenously processed and presented for recognition by cellular immunity.

The FBP-derived peptides shown in this study to be recognized by ovarian cancer-associated CTL may be utilized in several different immunotherapeutic strategies. First, these peptides could be used to stimulate FBP-specific CTL in vitro for cellular therapy. Adoptive immunotherapy has been shown to significantly reduce tumor burden in up to 30% of end-stage melanoma and

renal cell carcinoma patients with TIL (37, 38). And in ovarian cancer, TIL in combination with chemotherapy had a synergistic effect with better results than chemotherapy alone (39). These results were with uneducated and largely nonspecific TIL, and could be appreciably improved with highly specific CTL directed toward a known TAA. CTL induction studies with FBP peptides are currently underway in our laboratory.

These peptides may also form the basis of a peptide vaccine. The delivery systems for peptide antigens including dendritic cells and viral vectors are currently being investigated in melanoma with several ongoing studies (40). Encouraging results have been reported with the efficient induction of cellular responses in vivo to melanoma-derived peptide antigens effectively delivered (41, 42). We, too, have been investigating HER2/neu-derived peptides in similar strategies and have found efficient CTL induction in vitro with peptide-pulsed dendritic cells (43). We are currently studying the E75 peptide in clinical trials and virally delivered peptide in vitro. Similar studies will soon be initiated with FBP-derived peptides.

The success of vaccine development against epithelial cancer rests on the identification of widely expressed, CTL-recognized antigens that are either exclusively or highly associated with cancerous cells. FBP appears to be the second such known antigen and may be superior to HER2/neu given its distribution and level of expression as a target for cellular immunity.

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FIGURE LEGENDS

FIGURE 1- Freshly cultured ovarian TAL recognize FBP peptides. Consecutive HLA-A2$^+$ ovarian TAL (OvTAL) were isolated from malignant ascites and cultured in IL-2 without specific stimulation. OvTAL1 and OvTAL4 were tested at multiple E:T ratios in standard 5 hr chromium-release assays for recognition of the HLA-A2$^+$, antigen processing mutant, T2 (16), when loaded with five FBP peptides (E37-E41) or no peptide (T2) as a negative control.

FIGURE 2- FBP peptide recognition by consecutive ovarian TAL. Four consecutive HLA-A2$^+$ OvTAL populations were tested against T2 loaded with the five FBP peptides in standard 5 hr cytotoxicity assays. These assays were performed in triplicate at an E:T ratio of 20:1 and replicated for each effector. The results are expressed as % specific lysis $\pm$ SEM.

FIGURE 3- OvTAL recognition of FBP peptide-loaded T2 is HLA-A2-restricted. mAb blocking assays were performed with the anti-HLA-A2 mAb, BB7.2, and the anti-HLA class I mAb, W6/32. **(A)** OvTAL2 was tested against T2 loaded with E39, E37, or no peptide in standard 5 hr cytotoxicity assays at multiple E:T ratios. The specific lysis of T2/39 was inhibited by adding BB7.2 to the wells 30 min prior to the assays. **(B)** Both BB7.2 and W6/32 were tested for inhibition of the cytotoxicity of OvTAL2 and OvTAL4 for T2/39 in 20 hr assays at multiple E:T ratios. T2 loaded with no peptide or E37 were used as negative controls. Results are expressed as % specific lysis. Results were confirmed in multiple assays.

FIGURE 4- OvTAL recognition of FBP peptide, E39, is concentration dependent. OvTAL1 was tested against T2 incubated with increasing

concentrations of peptide in standard 5 hr cytotoxcity assays at an E:T ratio of 20:1. The results are expressed as % specific lysis ± SEM.

FIGURE 5- The FBP peptide, E39, is a naturally processed and presented antigen in ovarian cancer. Cold target inhibition assays were performed with OvTAL1 and OvTAL4 in replicated experiments. Cold T2 loaded with E37 (negative control peptide), E39, or no peptide (T2) were tested at a cold:hot ratio of 15:1 for inhibition of the recognition of the ovarian cancer cell line, SKOv3.A2 (27) by OvTAL at E:T ratios of 20:1 in 20 hr chromium-release assays. Results are expressed as % inhibiton by T2/39 compared to T2/37 and T2.

Table I- FBP-derived peptide sequences and HLA-A2 stabilization assays to confirm peptide binding and relative affinities.

FBP Peptide	Sequence	HLA-A2 (MCF ratio)*
E37 (25-33)	RIAWARTEL	1.16
E38 (112-120)	NLGPWIQQV	3.64
E39 (191-199)	EIWTHSTKV	1.33
E40 (247-255)	SLALMLLWL	1.18
E41 (245-253)	LLSLALMLL	1.20
No Peptide		1.00

*MCF=mean channel fluorescence of FACS analysis with anti-HLA-2 mAb, BB7.2. No peptide was utilized as baseline expression of HLA-A2 on T2. Results are expressed as a ratio of the MCF with the specific peptide as compared to no peptide.

FIG 1

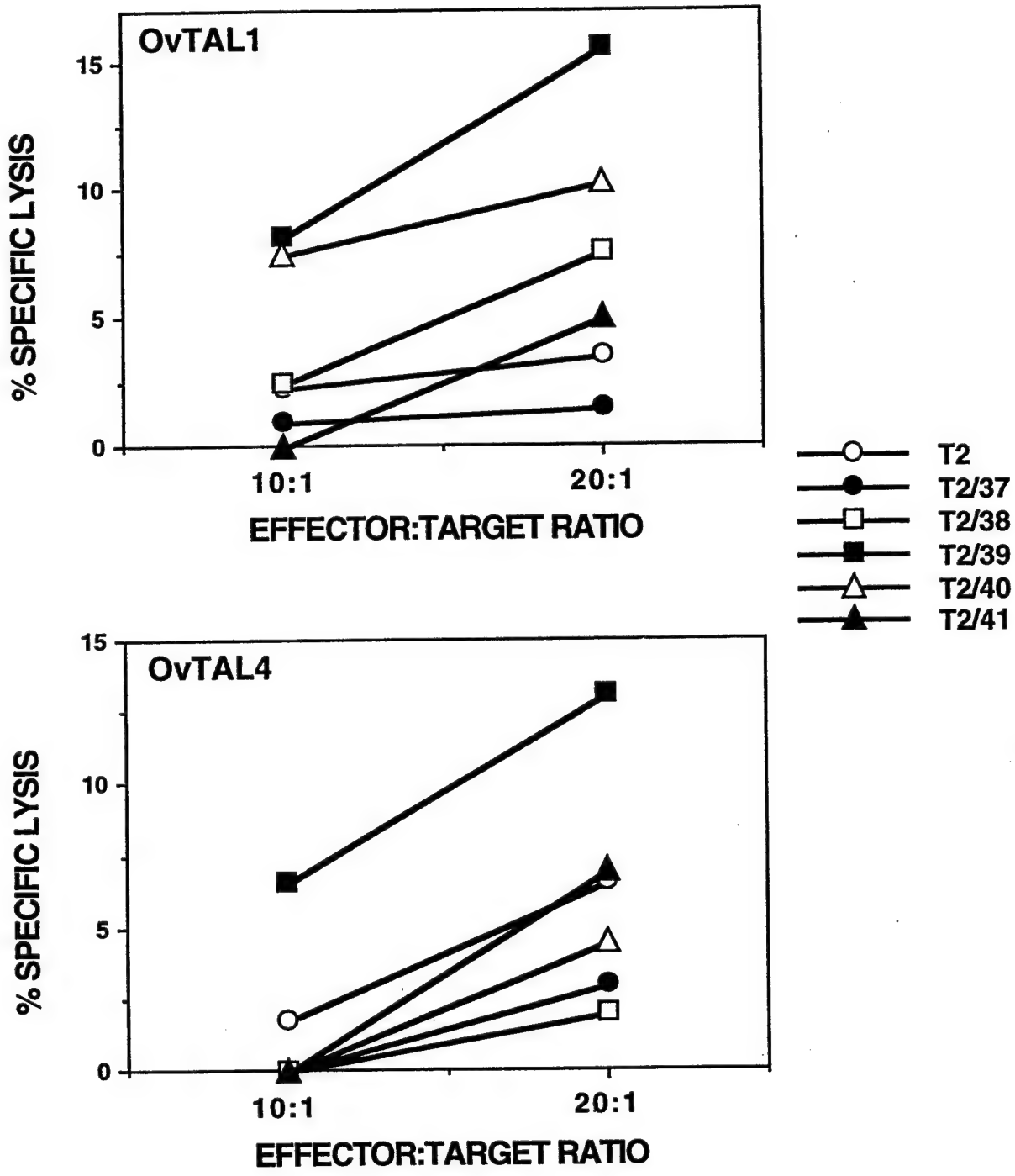

OvTAL1
% SPECIFIC LYSIS
15
10
5
0
10:1
20:1
EFFECTOR:TARGET RATIO
T2
T2/37
T2/38
T2/39
T2/40
T2/41
OvTAL4
% SPECIFIC LYSIS
15
10
5
0
10:1
20:1
EFFECTOR:TARGET RATIO

FIG 2

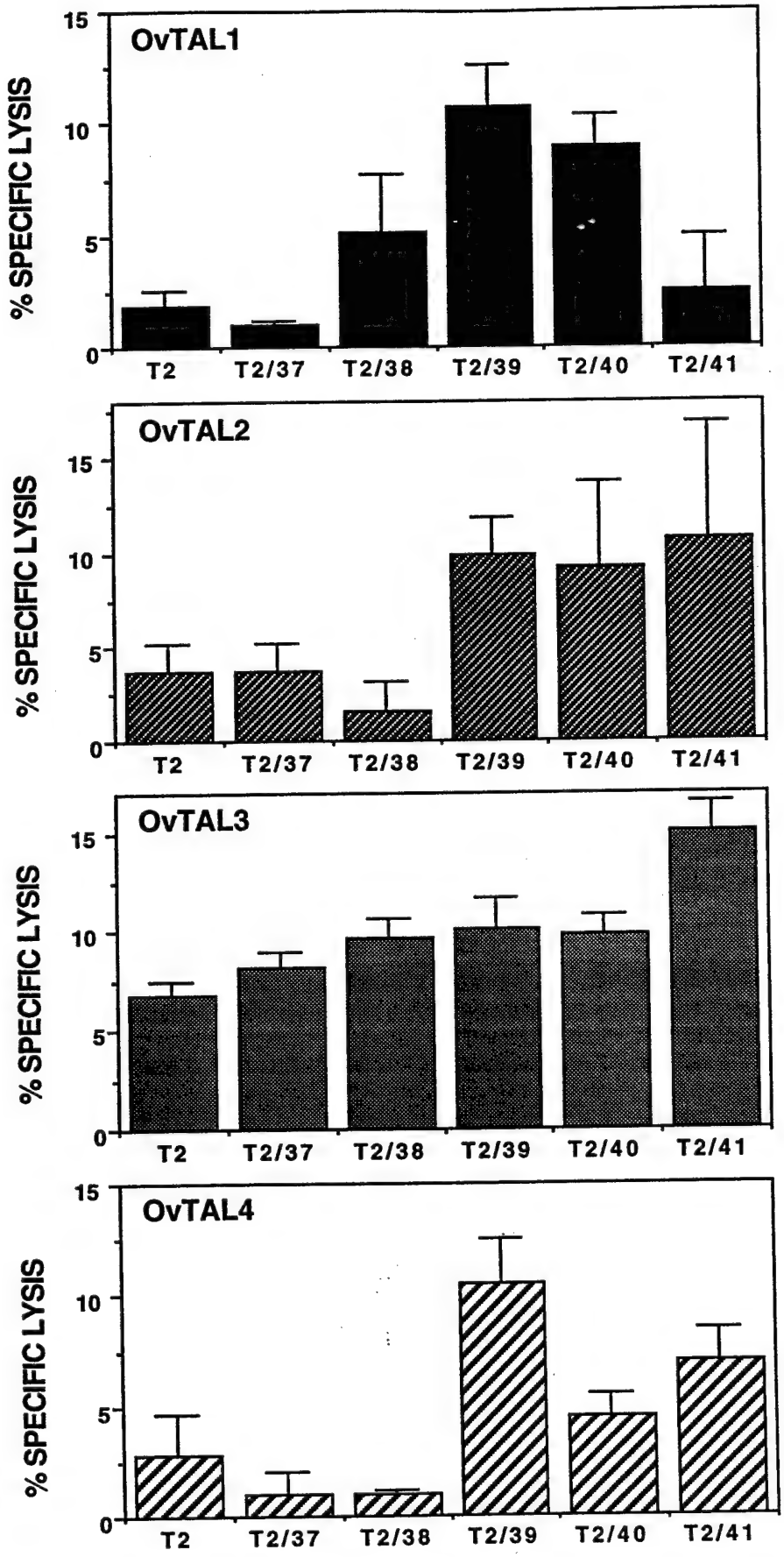

OvTAL1
OvTAL2
OvTAL3
OvTAL4
% SPECIFIC LYSIS
15
10
5
0
T2
T2/37
T2/38
T2/39
T2/40
T2/41
PEPTIDE-LOADED T2

FIG 3A

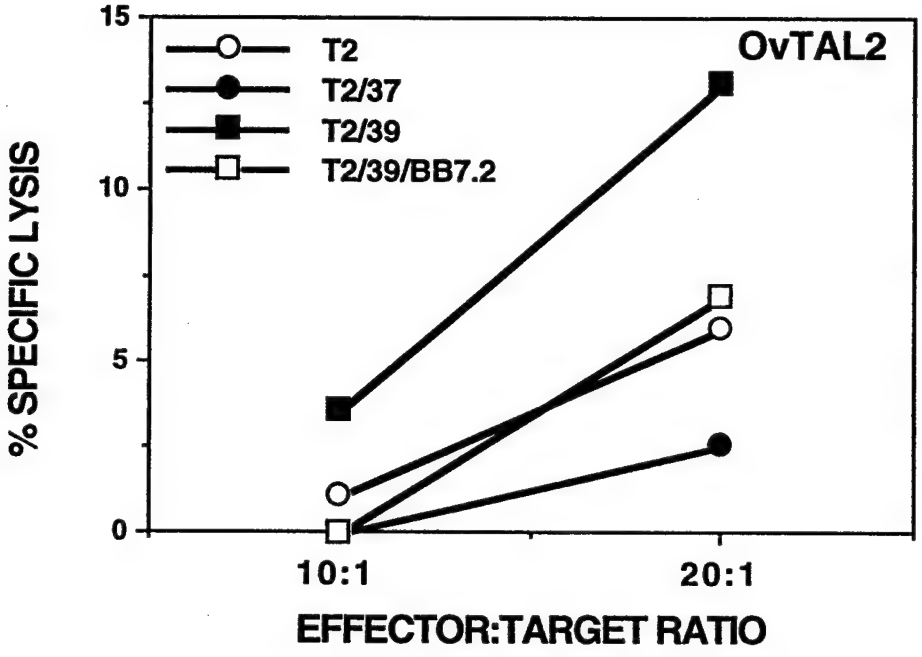

15
10
5
0
% SPECIFIC LYSIS
T2
T2/37
T2/39
T2/39/BB7.2
OvTAL2
10:1
20:1
EFFECTOR:TARGET RATIO

FIG 3B

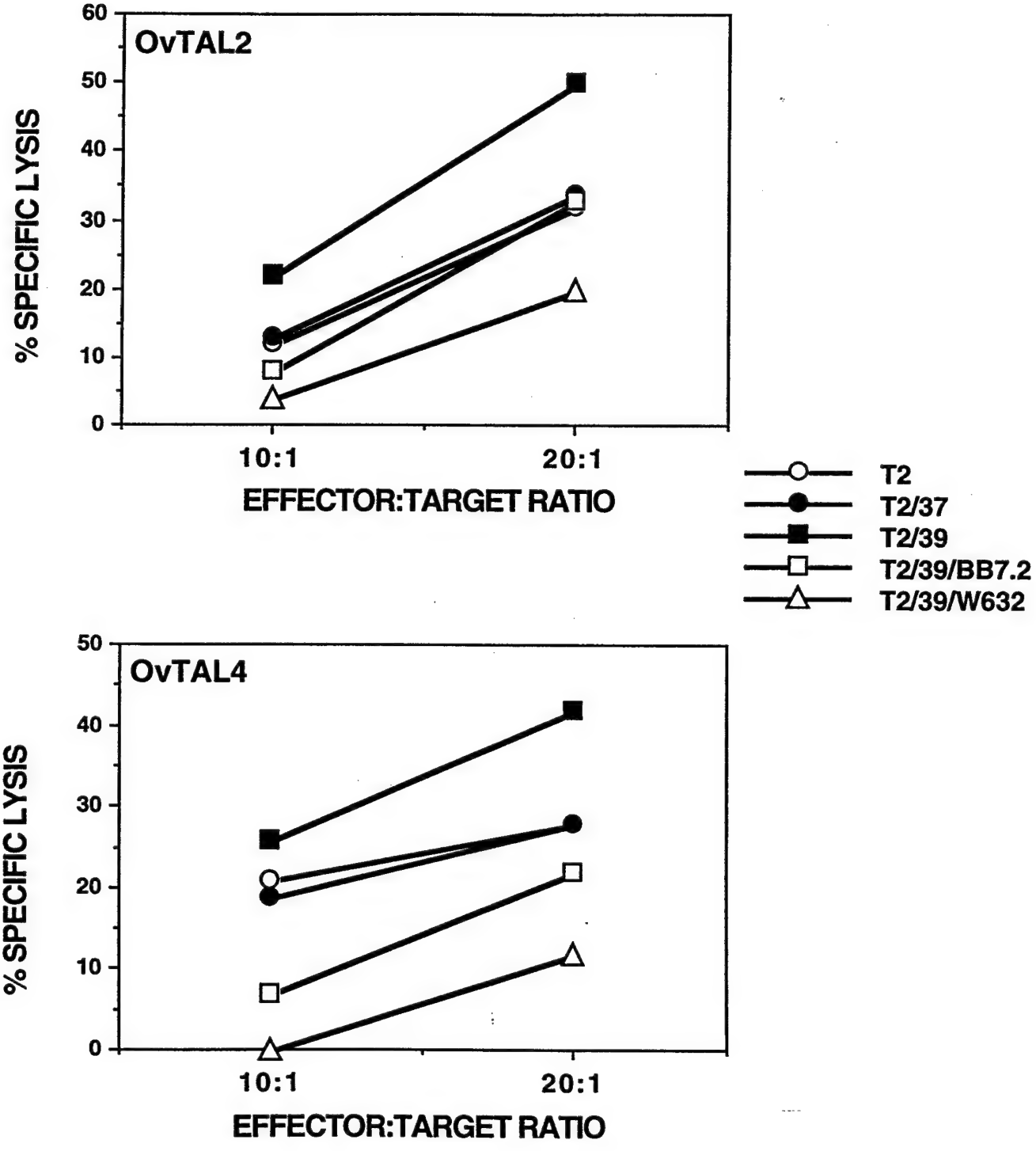

OvTAL2
% SPECIFIC LYSIS
60
50
40
30
20
10
0
10:1
20:1
EFFECTOR:TARGET RATIO
T2
T2/37
T2/39
T2/39/BB7.2
T2/39/W632
OvTAL4
% SPECIFIC LYSIS
50
40
30
20
10
0
10:1
20:1
EFFECTOR:TARGET RATIO

FIG 4

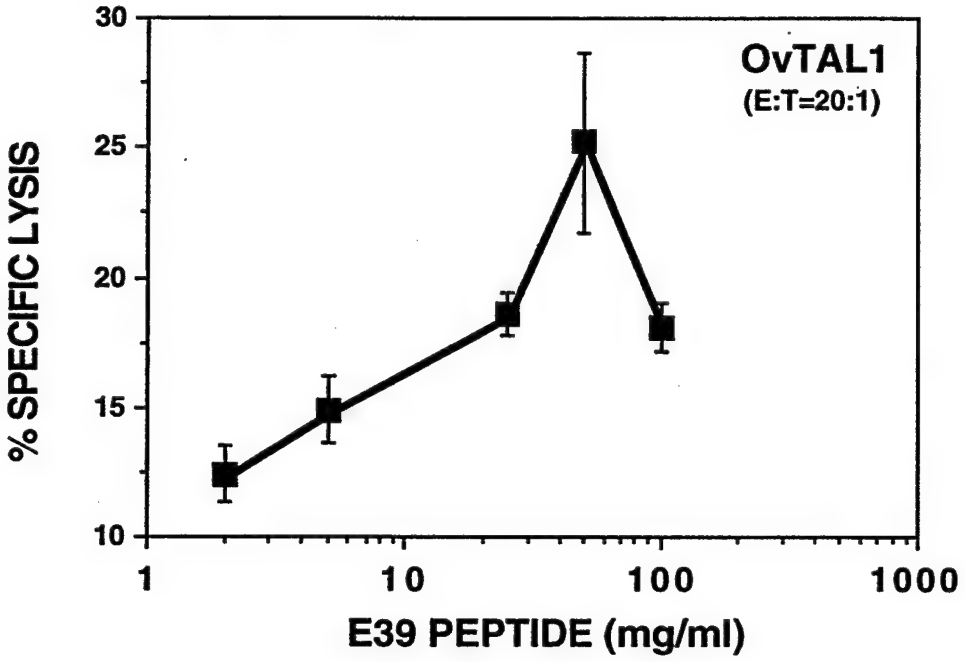
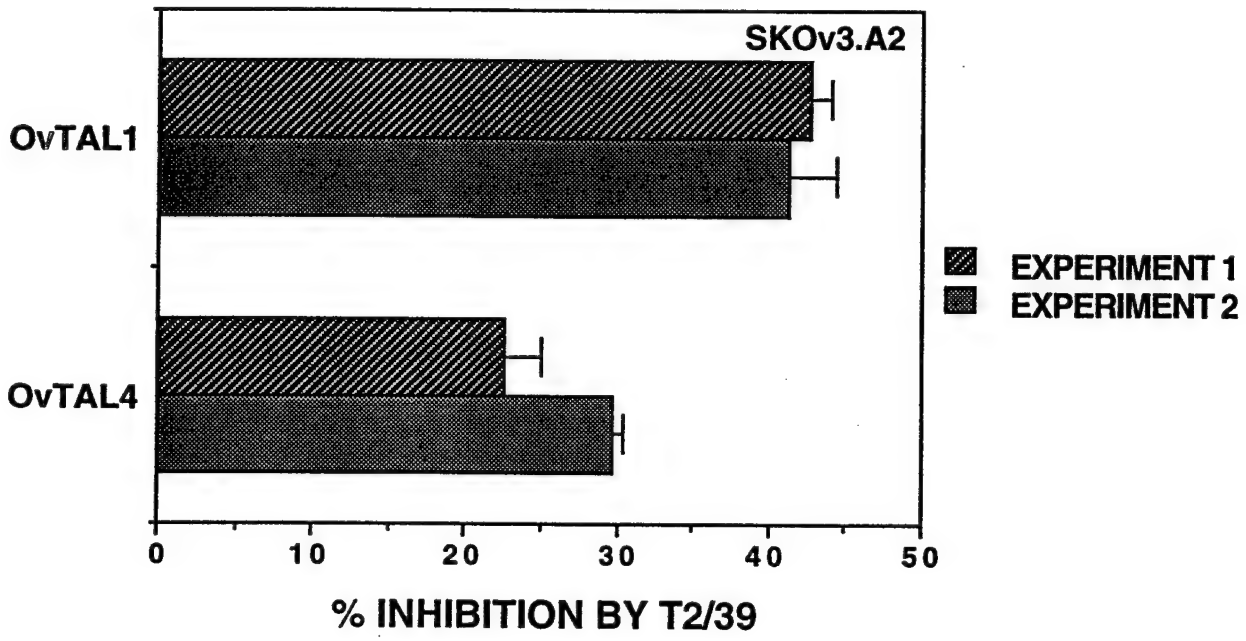

30
25
20
15
10
% SPECIFIC LYSIS
OvTAL1
(E:T=20:1)
1
10
100
1000
E39 PEPTIDE (mg/ml)

FIG 5


SKOv3.A2
OvTAL1
OvTAL4
EXPERIMENT 1
EXPERIMENT 2
0
10
20
30
40
50
% INHIBITION BY T2/39

Rapid activation of CTL effector functions by HER-2 peptides reveals functionally distinct populations in healthy individuals.

Brett W. Anderson, George E. Peoples, J. Taylor Wharton and Constantin G. Ioannides

Departments of Gynecologic Oncology and Surgical Oncology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030

Running Title: Differential tolerance in HER-2 specific CTL

Key words: dendritic cells, CTL, peptide, HER-2, interferon-γ, tolerance, IL-12, CTLA-4

Abbreviations used in this paper: DC, dendritic cells; HER-2, HER-2/neu protooncogene

Please address correspondence to: Dr. Constantin G. Ioannides, The University of Texas M.D. Anderson Cancer Center, Department of Gynecologic Oncology, 1515 Holcombe Blvd., Box 67, Houston, Texas 77030, Tel: 713-792-2849, Fax: 713-792-7586.

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Summary

Induction of CTL effector functions is thought to result from Ag recognition on antigen presenting cells, and it is characterized by $CD8^+$ cells proliferation and differentiation to effectors capable of mediating specific lysis and cytokine secretion upon encounter with the stimulating Ag. We examined the ability of a HER-2 peptide, E75, mapping an immunodominant CTL epitope for ovarian and breast tumor associated lymphocytes (TAL), in activating CTL effector functions in peripheral $CD8^+$ cells from healthy individuals. IFN-γ was rapidly induced in an E75 concentration dependent fashion, in the presence of IL-12, in all seven donors even within 6 h of Ag exposure, but only in two donors in the absence of IL-12. MAb to B7.1, B7.2 and CTLA-4 synergized with IL-12 in amplifying the IFN-γ response to E75 in all donors. The major source of IFN-γ appeared to be *ex vivo* activated $CD8^+$ memory cells. Conversely, IL-12 and blocking of CTLA-4/B7.1 promoted only marginal proliferation of $CD8^+$ cells to E75, and did not induce/enhance antigen–specific lysis, neither in the donors with existent $CD8^+$ cells of E75 specificity (three of eight) nor in the five healthy individuals where such specificity was absent. The presence of functionally distinct, partially tolerized effectors, supports the existence of a diverse clonal composition dominated by different subpopulations in healthy individuals. Some of these cells may play an early role in protective immunity to the onset of IFN-γ sensitive HER-2 tumors.

Introduction

The role of $CD8^+$ CTL in development of responses to malignant diseases has been emphasized by recent findings. Antigens recognized by melanoma, ovarian and breast CTL have been recently identified and demonstrated to be self-proteins (1). The fact that these CTL co-exist with progressing tumors (2) suggest that presentation of Ag by tumors or existent APC cannot elicit the biological functions in CTL (3), at the levels required to mediate an effective anti-tumor response. Recently it has been proposed, and demonstrated in model studies that the induction of tumor specific CTL effector functions could be accomplished by using dendritic cells (DC) as APC (4). DC have been reported to exert both immunostimulatory and tolerogenic effects for both naïve and memory T cells as well as to induce/re-activate CTL, suggesting a dependency of their function on the source ($CD34^+$ or $CD14^+$ precursors), and the cytokine environment used for their maturation and expansion (4-8).

There is little information on the ability of human DC to activate effector functions by CTL specific for human tumor Ag (self Ag). Activation of cytotoxic function of CTL specific for MART-1 and HER-2 Ag have been reported for DC derived from $CD34^+$ and $CD14^+$ precursors in some systems (9-11). It is unknown whether HER-2 specific CTL were present in the donors tested, and were amplified at restimulation, or *in vivo* priming by tumor induced functional E75 specific effectors. Induction/enhancement of tumor specific CTL activity required a minimum of 4-5 *in vitro* stimulations over 4-6 weeks and various

cytokine combinations to promote responder growth (9-11). This raises the question whether the activation of autonomous proliferation and induction of specific lytic function in peripheral $CD8^+$ cells responding to self-antigens is dependent on the presence of activated effectors or whether the responders are tolerized.

For understanding the immunity to cancer, and vaccine development, it is important to identify the $CD8^+$ cell effector functions that are activated by different Ag-APC combinations. To address these questions we used as APC, DC generated by the $CD14^+$ method (4, 12), i.e. from plastic-adherent cells of peripheral blood mononuclear cells (PBMC), because they could elicit CTL specific for tumor peptides (9-11), to stimulate HER-2 specific CTL from human blood. This model was chosen because HER-2 over-expression ($HER\text{-}2^{hi}$) in ovarian, breast and lung tumors is associated with poor prognostic and survival in these cancers. Control of growth of $HER\text{-}2^{hi}$ tumors is not achieved through conventional therapeutic modalities. However, $HER\text{-}2^{hi}$ tumors are sensitive to IFN-γ, which inhibits their proliferation and HER-2 over-expression (13). HER-2 can be also used to understand induction of immunity to other over-expressed tumor proteins in the same epithelial tumor system, e.g. Muc-1, folate binding protein, (FBP) (14) or other e.g. melanoma gp100 systems.

Based on these considerations, we investigated the ability of a peptide, E75, mapping an immunodominant CTL epitope, HER-2 (369-377) (15) to stimulate

$CD8^+$ cells from healthy donors, when pulsed on $CD13^+$ CD14 - DC (DC-E75). DC-E75 stimulated high and rapid Ag specific IFN-γ secretion by these $CD8^+$ cells, only in a fraction of donors (two of seven tested). This response was amplified by IL-12 in all seven donors, and in most instances further enhanced by mAb to B7.1/B7.2 and CTLA-4 molecules. Primary DC-E75 stimulation in the presence or absence of IL-2, TNF-α and/or IL-12 did not enhance the specific cytotoxicity in the same cells. For all donors, DC-E75 stimulation increased only slightly the $CD8^+$ cell proliferation, suggesting that the responders may be partially tolerized.

Materials and Methods

Cells. HLA-$A2^+$ PBMC were obtained from healthy volunteers from the Blood Bank of M.D. Anderson Cancer Center. The HLA phenotype of the donors used in this study are as follows: Donor 1 (A2, B7, 44), Donor 2 (A2, 33, B40, 44), Donor 3 (A2, 33, B41, 81), Donor 4 (A1, 2, B27, 44), Donor 5 [A1, 2 B44, 57, Cw5, 6), Donor 6 (A2, 31, B35, 44, Cw4, w5), Donor 8 (A2, 24, B27, 51). For Donor 7 only the presence of HLA-A2 was confirmed. DC from HLA-$A2^+$, HER-2^{hi} breast cancer patient #127 were obtained after G-CSF induced $CD34^+$ mobilization. $CD34^+$ and $CD14^+$ were purified by a magnetic cell separation system. T2 cells, ovarian SKOV3 and SKOV3.A2 cells, tumors and indicator tumor associated lymphocytes (TAL) from ovarian ascites were described (15).

Cytokines. The following cytokines were used in this study: GM-CSF (Immunex Corp., Washington, DC), specific activity 1.25 x 10^7 CFU/250 mg, TNF-

α (Cetus Corp., Emeryville, CA), specific activity 2.5 x 10^7 U/mg, IL-4 (Biosource International), specific activity, 2 x 10^6 U/mg, IL-2 (Cetus Corporation), specific activity 18 x 10^6 IU/mg. IL-12 of specific activity 5 x 10^6 U/mg was a kind gift from Dr. Stanley Wolf, Department of Immunology Genetics Institute.

Synthetic peptides. The HER-2 peptides used were: E75 (369-377) its mutated analogs: F41 (GIFGSLAYL) and F46 (KIFGSLAYL), GP2/F53: (IISAVVGIL, 654-662) (16), and F57: (IHLNGSAYSL, 439-447) (17). Peptides GP2 and F57 define HER-2 CTL epitopes distinct from E75 (16, 17). The modified Muc-1 peptides used were D125: (GVTSAKDTRV) and D132 (SLADPAHGV). The folate binding protein (FBP) peptides used were: E39 (FBP, 191-198) EIWTHSTKV, and E41 (FBP, 245-253) LLSLALML. These FBP peptides were recently identified to be recognized by ovarian and breast TAL (14). All peptides were prepared by the Synthetic Antigen Laboratory of M.D Anderson Cancer Center and purified by HPLC. Peptides were 95-97% pure by amino acid analysis. Peptides were dissolved in PBS and stored frozen at -20° C in aliquots of 2 mg/ml.

Immunofluorescence. Antigen expression by DC, T2 cells, and T cells were determined by FACS using a flow-cytometer (EPICS - Profile Analyzer, Coulter Co, Hialeh FL). mAb to CD13 and CD14 (Caltag Laboratories, San Francisco, CA), B7.1 and B7.2 (CD80 and CD86, Calbiochem), ICAM-1 (CD54, Calbiochem), CD40L (Ancell, Bayport, MN), HLA-A2 (clone BB7.2, ATCC), and MHC-II (L243,

DAKO Corp., Carpintera, CA) were used as FITC or PE conjugated. DC cells were defined by the presence of CD13 and absence of CD14 marker after culture in GM-CSF and IL-4. For phenotype analysis, DC were incubated at 4°C with PE-conjugated anti CD13 mAb and FITC-conjugated anti CD14 mAb for 30 minutes. Positively stained cells were measured by flow cytometry. For determination of the effects of cytokines, peptides and T cells on the surface antigens expression, DC were incubated with the same amounts of cytokines and peptides as in CTL induction assays for 24 h, and the levels of Ag expression were determined in the gated $CD13^+$ population. For determination of the $CD8^+$ blast formation, cells cultured under various CTL induction conditions were stained with FITC conjugated OKT8 mAb and examined in the flow-cytometer by forward-scatter analysis (FSA) as described (18).

Culture of peripheral blood mononuclear cells. $CD13^+$ DC were generated from freshly isolated PBMC with some modifications of the established CD14 methods (12, 19). PBMC were suspended at 2×10^6/ml were distributed at 4×10^6 cells/well in 24 well plates in RPMI 1640 without FCS. After 2 h incubation at 37°C, the non-adherent cells were removed by gentle repeated aspiration and stored frozen in liquid nitrogen. $CD8^+$ cells were isolated by removing the $CD4^+$, the $CD16^+$ and the $CD56^+$ cells from the non-adherent population using Dynabeads (Dynal, Oslo, Norway). Removal of activated $CD8^+$ cells was performed using anti CD45RO mAb (UCHL-1, DAKO) as described (20). Typically after depletion, the resulting cells were more than 85% $CD8^+$ as determined by

flow cytometry. Complete RPMI medium (containing 10% FCS) supplemented with 1000 U/ml GM-CSF or 500 U/ml IL-4 was added to each well containing plastic adherent cells and maintained for eight days. This combination of serum and cytokines were found in pilot experiments to optimally support induction of CD13 and up-regulation of CD40 and MHC-II on DC compared with GM-CSF, GM-CSF+TNF-α, and GM-CSF+TNF-α+IL-4 tested in parallel in RPMI containing either FCS, or HS or AIM-V medium. DC proliferation was borderline, 10-20% maximum increase in cell number over an 8-day period, (data not shown). This was in agreement with recent reports indicating that DC generated from $CD14^+$ PBMC showed only signs of proliferation (21).

T cell stimulation by peptide pulsed DC. DC were washed three times with serum free medium, plated at 1.2×10^5 cell/well in 24-well culture plates and pulsed with peptides at 50 μg/ml in serum free medium for 4 h before addition of responders. In certain experiments, TNF-α (50 U/ml) was added to DC for the last 60 min to stimulate Ag uptake and presentation 1 (12, 19) and maintained in cultures during the entire stimulation period. Autologous non-adherent PBMC, isolated $CD8^+$ cells or isolated $CD8^+$ cells ($CD45RO^+$ cells depleted) in RPMI 1640 containing 10% HS were added to DC at 3×10^6/well (stimulator:responder ratio of 1:25). 3 U of IL-12 were also added to the corresponding wells since it was separately determined that this is the lowest concentration potentiating IFN-γ induction by E75. 16 h later IL-2 was added to each well at a final concentration of 5 Cetus U/ml and the cultures were left undisturbed for the following 5 days

when CTL activity was determined. In separate experiments we determined that this approach was more effective in elicitation of CTL activity to E75 than higher S:R ratios (Anderson et. al. preliminary results). For functional studies mAb specific for B7.1, B7.2, HLA-A2, in the amounts reported to be inhibitory by the manufacturers were added to DC, 1 h before responders. Anti CTLA-4 was added to T cells for 1 h before they were added to cultures. The effects of peptides and cytokines on T cells survival were determined by counting the numbers of recovered viable cells (22) in parallel with determination of the numbers of $CD8^+$ and $CD4^+$ cells in the sample by flow-cytometry. Specific proliferative responses to E75 in the presence or absence of cytokines were determined by measuring the incorporated radioactivity in equal cell numbers pulsed with 1 μCi of (^{3}H)-TdR (23).

CTL and cytokine assays. Recognition of peptides used as immunogens by CTL was performed as described (15). Effectors from each well containing different cytotokine combinations were counted. Equal numbers of viable effectors were used in all assays. Unnatural (mutated) Muc-1 peptides were used as indicators of cross-reactive recognition. 50 μl supernatants collected at 24-48 h were tested in duplicate for the presence of IL-2, IL-4, and IFN-γ using cytokine ELISA-kits -(Biosource International, Camarillo, CA) as described (23). The sensitivity of the assay was 4 pg/ml. The amount of cytokines was quantitated using a standard plot of known concentrations of cytokines determined in the same experiment.

Results

$CD8^+$ cells from healthy donors display specific IFN-γ secretion within 6-24 h of contact with HER-2 peptides. Potentiation by IL-12 and anti B7/CTLA-4 antibodies. $CD8^+$ cells responses are induced by presentation of Ag by MHC-I molecules of antigen presenting cells (APC). For characterization of the ability of Ag and APC to stimulate T cell activation, analysis of several biological responses is important. Two of these responses, induction of cytokine secretion and induction/ amplification of specific cytotoxic function define the main effector functions of $CD8^+$ T cells (3). The third, induction of $CD8^+$ cells proliferation and clonal expansion, define their ability to produce their own growth factors, increase their responsiveness to exogenous IL-2 and ultimately the "strength" of the response (3, 18, 24, 25). To study the activation of T cells by a HER-2 (self)-peptide presented by $CD14^+$ derived DC cells we used T cells from several HLA-$A2^+$ healthy donors to assure that the conclusions are relevant. Although the presence of specific effectors for the HER-2 peptides E75 (369-377) and GP2 (654-662) in cancer patients has been documented (15-17), information is lacking about the presence and modalities of activation of T cells responding to these peptides. We also rationalized that if in healthy donors $CD8^+$ cells can be stimulated by a tumor Ag presented by DC, this may be of interest for cancer vaccination.

We used as APC, $CD13^+$ DC, because such cells have been reported to activate both naive and memory CTL (4, 26) and present E75 (10). After culture

in GM-CSF+IL-4, DC from all donors were >96% $CD13^+$, CD14-expressed high levels of MHC-I, MHC-II, CD54, CD40, and CD86, but lower levels of CD80 in agreement with the recently described phenotype of peripheral blood $CD14^+$ derived DC (19, 21). The levels of expression of CD80 and CD86 on DC were significantly lower than on T2 cells. 16 h incubation of DC with E75 lead to increased levels of HLA-A2 (MCF, DC-NP=15.6, DC-E75=27.0) suggesting that E75 stabilized HLA-A2. DC increased MHC-II expression after addition of E75 and T cells. In DC from three donors tested (1, 4 and 6) neither cytokines, nor T cells, nor E75, not their combinations induced significant up-regulation of B7.1 within the first 24 h (MCF:DC-NP=2.8, DC-E75,3.0) and B7.2 (MCF:DC-NP=10.7, DC-E75=11.9) compared with CD40 (MCF:DC-NP=13.3, DC-E75=30.5). TNF-α+ IL-12 at the concentrations used for IFN-γ and CTL induction (2 pg/ml and 330 pg/ml respectively) did not induce a significant up-regulation of these markers on DC within the first 24-48 h of incubation (Anderson et. al. manuscript in preparation).

We determined in parallel the ability of this Ag-APC system to induce rapid cytokine secretion (INF-γ and IL-2), specific cytotoxic function, and T cell proliferation. An important feature of the determination of cytokine secretion is that responses of T cells can be measured rapidly and that it was possible to detect changes in functional cytokine response patterns within the first 24 h of Ag exposure.

To determine whether the cytokine responses are Ag specific we determined the levels of IFN-γ secreted by unseparated T cells from Donor 1, in response to E75, and the recently identified FBP CTL epitope E39 (14) **(Fig. 1A).** E75 presentation on DC induced T cells to secrete low levels of IFN-γ within 24h. The presence of IL-2 for the last 8h in culture did not increase IFN-γ secretion (≤20 pg/ml). IL-12 (330 pg/ml) dramatically increased IFN-γ secretion, above the levels observed with IL-12 alone, or IL-12 plus control unnatural Muc-1 peptide D132. IL-2 and IL-4 were not detected in the E75 stimulated T cell cultures from this donor (data not shown). Since the IFN-γ secretion appeared to be Ag specific, we investigated whether B7 mediated co-stimulation interfered with this response. Since B7.1 and B7.2 were expressed on DC at different levels, their functions were described to be either similar or opposite, and B7 can engage two different receptors on T cells (CD28 and CTLA-4) with distinct outcomes (27, 28), we used alternatively anti B7.1 and anti B7.2 mAb to block the receptors ligation. The results show **(Fig. 1B)** that even in the absence of IL-12, blocking of B7.1 and B7.2 enhanced the IFN-γ response, supporting similar functions, but the effects were higher following blocking of B7.1. These results were confirmed with Donor 6 using as APC T2 cells which express several fold higher levels of B7.1 and B7.2 than DC (not shown). We found that by blocking B7.2, induction of IFN-γ by E75 in the absence of IL-12 was E75 concentration dependent. 0, 1 and 50 μg/ml E75 induced secretion of: 0, 250 and 550 pg/ml of IFN-γ respectively within 24 h. This experiment was repeated with Donor 2 in the absence of exogenous IL-2, but in the presence of TNF-α **(Fig. 1C).** TNF-α has been shown to enhance the Ag

presenting ability of DC (10, 19). In the absence of IL-12, E75 induced high IFN-γ secretion, which appeared to be partially inhibited by TNF-α. The potentiating effect of IL-12 in IFN-γ induction was dependent on the Ag. This is illustrated by the fact that the IFN-γ levels were significantly higher with E75 than with F57 of higher binding and stabilizing activity.

To distinguish whether the rapid IFN-γ secretion together with the lack of IL-2 secretion are a property of activated $CD8^+$ cells and to verify that the observed responses are not limited to only two donors, $CD8^+$ cells from Donor 3 were stimulated with DC-E75 in the presence or absence of IL-12. Rapid induction of IFN-γ by E75 within 6 h was observed in cultures containing 3 U IL-12 **(Fig. 2A)**. The levels of IFN-γ continued to increase over the next 48 h **(Fig. 2B)**. At this time, IFN-γ was detectable even from the cultures that did not receive IL-12, although the levels of IFN-γ were significantly lower than in cultures that received IL-12. We confirmed that the levels of IFN-γ are dependent on the Ag sequence and/or the existent responder clonal size, since FBP peptides, E39 and E41, mapping immunodominant and subdominant CTL epitopes respectively, showed distinct abilities to induce IFN-γ during the same interval. Secretion of IFN-γ was Ag concentration dependent, confirming that E75 specific T cells and not NK cells were the source of this cytokine **(Fig 2C)**. Similar results were obtained with the subdominant HER-2 epitope GP2 (data not shown). Since the rapid IFN-γ secretion suggested that the responders may be in an activated state, and B7 ligation by CTLA-4 may transmit a negative signal to CD8+ cells, the

experiment was repeated in the presence and absence of anti CTLA-4 mAb. The results in **Fig. 2D** show a synergistic effect between IL-12 and anti-CTLA-4 in IFN-γ induction, within 24 h, in response to E75. The high levels of IFN-γ in response to E75 declined by 5 fold when the CD45RO+ cells were removed from CD8+ cells, suggesting that the main IFN-γ producers were *ex vivo* activated CD8+ cells.

To confirm that the effects of E75 and IL-12 are directed to $CD8^+$ cells, and identify additional patterns of response the experiment was repeated with isolated $CD8^+$ cells from Donor 4. The results **(Fig 3A)** confirmed that high IFN-γ secretion within the first 24 h required both E75 and IL-12. TNF-α affected only slightly IFN-γ secretion. With regard B7.1 and B7.2 inhibitory function, the results obtained with $CD8^+$ cells **(Fig. 3B)** were similar with the results obtained with unseparated T cells from Donor 1, suggesting that blocking of B7 ligation enhanced IFN-γ induced by E75. The enhancing effects of anti B7.1 in IFN-γ secretion in the first 24 h required the presence of IL-12 **(Fig 3B)** suggesting individual dependent quantitative but not qualitative differences in B7 function. The potentiating effects of IL-12 on IFN-γ secretion were higher when IL-12 was added to unseparated T cell cultures together with the responders **(Fig. 3C, line s)**. When IL-12 was added to the DC, 2 h before the responders, the levels of IFN-γ secreted were lower **(Fig. 3C, line e)**, IFN-γ secretion was not observed when IL-12 was added to the cultures 12 h later **(line l)** suggesting that T cells, E75, and IL-12 need to be present for maximal IFN-γ induction.

The levels of E75 induced IL-2 at 24 and 48 h in the same experiment **(Fig. 3B)** were low and only slightly different from the IL-2 levels in the absence of peptide: 40 pg/ml vs 36 pg/ml (NP). When IL-2 at 500 pg/ml was added for the last 8 h before supernatant collection to the cultures containing, TNF-α and IL-12 **(Fig. 3A)**, the amounts of IL-2 recovered at 24h were E75: 220 pg/ml and NP: 280 pg/ml. This suggested that E75 slightly enhanced the responsiveness to IL-2. The levels of IL-2 in the cultures containing E75+IL-2, E75+IL-2+TNF-α, and E75+IL-2+IL-12 **(Fig. 3A, lines 5, 4, and 3)** were 480, 420 and 240 pg/ml respectively suggesting that IL-12 enhanced consumption of IL-2. Similar results were obtained in the experiment shown in **Fig. 3C**, using unseparated PBMC when 1000 pg/ml of IL-2 were added for the last 8 h. The levels of recovered IL-2 at 24 h were as follows: *(a)* DC but no T cells: 853 pg/ml; *(b)* DC+T cells+TNFα+IL-12: 917 pg/ml indicating a slight increase in endogenous IL-2 production, *(c)* DC + T cells+TNFα+IL-12+E75:817 pg/ml, confirming E75 induced IL-2 consumption.

This experiment was repeated with isolated $CD8^+$ cells from the Donor 5 and the results were confirmed. Similar results regarding IFN-γ induction by E75 and potentiation by IL-12 and anti B7.1 were obtained with PBMC from a HLA-$A2^+$, HER-2^{hi} breast cancer patient (#127) suggesting that E75 presented on $CD14^+$ derived DC can rapidly activate IFN-γ secretion from T cells of both healthy donors and cancer patients (data not shown). Therefore, the IFN-γ response to tumor antigen peptides presented by DC is dependent on the nature of Ag in the

donor, it is amplified in all donors by IL-12 and by blocking of B7.1/B7.2 interaction with CTLA-4, and its derived primarily from $CD45RO^+$ cells.

The combination of IL-12 and IL-2 does not enhance specific cytotoxicity and T cell proliferation at primary stimulation with E75 pulsed DC. The rapid induction of IFN-γ secretion, even in the absence of IL-12 in certain donors, is suggestive of a pre-existent response to E75 consisting of activated and/or tolerized lymphocytes (20). To establish whether early activation of IFN-γ secretion is paralleled by activation of cytotoxic function we determined whether PBMC and isolated CD8 cells from the same donors, from the same cultures used to quantitate IFN-γ and IL-2, specifically recognized E75.

Since E75 re-stimulation of activated Ag-specific cells may lead to activation induced cell death (AICD), we determined in parallel the ability of cells stimulated with cytokines only (DC-NP) or E75 plus cytokines (DC-E75) to recognize E75 on T2 cells after five days of culture. Two distinct patterns of responses were observed: (1) one group of donors responded to both DC-NP and DC-E75 by similar E75 specific recognition (2) in the other donors E75 specific recognition was not detected after stimulation with DC-NP. In both groups DC-E75 stimulation failed to enhance specific recognition of E75 compared with recognition of cross-reactivity controls unnatural Muc-1 peptides **(Fig.4)**.

The first pattern of response was observed in Donor 1 **(Fig. 4A)** and was confirmed with Donors 2, **(Fig. 4B)** and 6 (not shown). Unseparated T cells stimulated with DC-NP or DC-E75 on a per recovered cell basis specifically recognized E75 at similar levels. E75 recognition was only slightly increased by stimulation with DC-E75 in the presence of TNF-α. In the presence of IL-12, stimulation with DC-NP lead to an increase in non-specific cytotoxic activity; stimulation with DC-E75 induced a slight, although not significant, decrease in E75 recognition but decreased the non-specific cytotoxic activity compared with stimulation with DC-NP **(Fig. 4A, B)**. The second pattern was more extensively characterized in Donor 4 **(Fig. 4C)** using isolated $CD8^+$ cells to minimize the contribution of $CD4^+$ and NK cells. On a per recovered cell basis isolated $CD8^+$ cells stimulated with E75 showed cross-reactive recognition by recognizing better the unnatural Muc-1 peptide D125, than E75. E75 + IL-12 enhanced both specific and non-specific recognition. Compared with $CD8^+$ cells that were stimulated with DC-NP plus TNF-α + IL-12, the increase in E75 specific recognition by DC-E75 was not significant **(Fig. 4C, line 3 vs. 4)**. mAb to B7.1 and B7.2 added to the cultures 30 min before addition of responders did not affect elicitation of E75 recognition, in the absence or presence of IL-2/IL-12 in the Donors 4 and 6, suggesting that induction of cytotoxic function in this system it is not B7 dependent (data not shown, also, **Fig. 5C** for Donor 3). Similar results were obtained with Donors 3, 5, 7, 8 and Patient 127. In fact, in this patient the use of $CD34^+$ derived DC as APC did not induce specific recognition of E75 (Anderson, Braunschweig, preliminary data).

To address whether the lack of enhancement in the cytotoxic function, reflects the lack of expansion of Ag stimulated cells, we determined the CD8 proliferation levels in response to E75 activation. The results in **Fig. 5A**, show that E75 stimulation of IFN-γ^+, CTL- Donor 3 $CD8^+$ cells ($CD45RO^+$, and $CD45RO^+$ depleted) induced only low levels of proliferation, regardless of the presence of IL-12, and anti CTLA-4 mAb (Calculated stimulation indexes varied between 1.5-2.0). Although IL-12, but not anti-CTLA-4, alone appeared to increase the rate of $CD8^+$ cells proliferation within 48 h, the increase in proliferation induced by E75 was marginal and similar in the presence or absence of IL-12. Thus, the effects of E75 on proliferation were different than on IFN-γ induction in the same system (shown in **Fig. 2D**). Although equal numbers of $CD45RO^+$ and CD45RO depleted cells were used for stimulation, and equal numbers of live recovered cells from both populations were incubated 48 h later with ^{3}H-TdR, the cpm values in the latter were only half compared with the former, indicating either a smaller frequency of E75 activated naïve cells or poor growth potential (20). Specific E75 recognition by $CD4^+$, $CD16^+$, and $CD56^+$ cells depleted responders stimulated with E75 and/or cytokines was not observed **(Fig. 5B)**, suggesting that preferential early activation of specific killer cells is not achieved in this system. The IL-2 levels from all combinations shown in **Fig. 5A, B** were 4-10 pg/ml, at both 24 and 48 h suggesting that even if low levels of IL-2 were secreted, they were rapidly absorbed and consumed. Similar results were obtained with the other donors when the numbers of recovered cells were compared after 5 days of stimulation. For example, in the presence of IL-2, TNF-a and IL-12, with a

designated stimulation index for live cells recovery of 1.0 for DC-NP, the values for DC-E75 were: 1.14 (Donor 1), 1.65 (Donor 2), 1.07 (Donor 4) and 1.64 (Donor 5), while for DC-E75 + anti B7.1 were: 1.31 (Donor 1) and 0.94 (Donor 4). Similarly, in the absence of IL-2, it the stimulation index for viable cell recovery was for Donor 5 DC-E75=1.0 but for DC-E75+IL-2=1.48±0.21, and DC-E75+IL-2+TNF-α=1.41±0.19 (average of three independent experiments). These results indicate that E75 responding cells in all healthy donors express a block in proliferation which is insensitive to B7-CD28 or B7-CTLA-4 interactions and cannot be corrected by exogenous IL-2 + IL-12. Similar lack of proliferation was observed when T cells from healthy donors and breast cancer patients were stimulated with E75 pulsed on plastic adherent PBMC or T2 cells in the presence or absence of IL-2 (Anderson, Murray, preliminary data). Therefore, E75-specific $CD8^+$ T cells are present in healthy donors and can be rapidly activated by E75 pulsed DC. These cells appear to contain distinct subpopulations in different proportions, based on their ability to express specific cytolytic function or not, and to require IL-12 to secrete IFN-γ. (Table I) A common feature of these cells is that E75 induce low (or undetectable) IL-2 and cannot stimulate these cells proliferation or recovery.

Discussion

The studies presented in this report were aimed at analyzing the ability of a tumor peptide E75, to activate effector functions in T cells freshly isolated from peripheral blood of healthy donors. Specifically, we analyzed the role of Ag, of a

prototypic inflammatory cytokine, IL-12 (29, 30), and of B7/CTLA-4 co-stimulation in these processes. In this system it was possibly to demonstrate that E75 specific $CD8^+$ cells are present in some donors (No. 2 and 3) in a state wherein they can secrete IFN-γ within 24 h of antigen exposure in the absence of IL-12 and in some donors (No. 1, 6 and 2) they can recognize E75 in the absence of Ag stimulation. The fact that significant IFN-γ release was triggered by exposure to the self-peptide in all non-responders in the presence of IL-12 suggests that distinct effectors dominate in each donor and these $CD8^+$ cells are not capable of immediate effector function in their natural state *in vivo*. We found synergy in IFN-γ secretion between Ag, IL-12, and blocking of B7 and -CTLA-4. This suggested that: *(a)* engagement of B7.1/B7.2 promoted negative signaling in the responder population; *(b)* B7 may engage its high affinity receptor CTLA-4 on $CD8^+$ cells with consequence tolerance induction. This indicates that predominant subpopulation of E75 responders may differ from primary activated or memory CTL described *in vitro* (20). The different CTL and IFN-γ response patterns did not reflect variations in the APC function of DC. DC from Donor 1, 2 and 4 (assigned to different response groups) responded similarly in terms of HLA-A2, CD40, CD54, CD80 and CD86 expression when incubated with E75, cytokines and T cells (Anderson et al. Manuscript in preparation). IL-12 treated showed similar capacity to trigger IFN-γ production by T cells in response to E75.

Indirect evidence that some of the cells secreting IFN-γ are Ag specific cytotoxic effectors is provided by the experiments in **Fig. 4** which shows that

PBMC from two donors recognize E75 even in the absence of peptide stimulation. These cells also lysed better SKOV3.A2 cells compared with SKOV3 cells (HLA-A2). Lysis of SKOV3.A2 cells was inhibited by T2-E75 suggesting that they recognize a naturally presented epitope (Anderson et. al. preliminary data). DC-E75 stimulation failed to enhance the specific cytotoxic activity present suggesting that E75 specific CTL did not expand.

Clonal expansion of CTL precursors is dependent on the responsiveness to IL-2. In this system, IL-2 was detected only in few donors, but at very low levels ranging between 15-40 pg/ml/$3x10^6$ cells in the first 24-48 h. When exogenous IL-2 was added to compensate for absorption or consumption, stimulation with E75 both in the presence and absence of IL-12 lead to decrease in the recovered amounts of IL-2 suggesting that more IL-2 is consumed than produced. This increase in responsiveness to IL-2 in the presence of IL-12 was confirmed in separated experiments by observing increased $CD8^+$ blast formation compared with IL-2 + TNF-α (data not shown). The low levels of IL-2 did not appear to be a consequence of the low levels of expression of B7.1 on DC, which would preclude engagement of CD28 on T cells, and/or of low Ag concentration. Similar low levels of IL-2 were observed when $B7.1^{hi}$, $B7.2^{hi}$, T2 cells were used as stimulators. The levels of E75 specific recognition by these responders were in the range of 10% at an E:T ratio of 20:1, and they were not enhanced by exogenous IL-2, or stimulation/restimulation with mutated E75 peptides F46 and F41 (not shown). Repeated stimulations of E75 primed CTL with DC-E75,

enhanced the specific CTL activity only in some (Donors 1 and 2) but not in other donors (3, 4, 7). This activity was short lived (7-10 days) and was prolonged but not enhanced by TNF-α. It is interesting to note that E75-specific CTL activity was present in the former but not the latter donors.

Our results indicate that within E75-specific memory $CD8^+$ cells similar with the ones recently reported for influenza (20) several subpopulations are present in healthy individuals and in some breast cancer patients. These cells appear to be in a state of partial tolerization/inactivation, requiring different stimulation thresholds to rapidly secrete IFN-γ. In some donors they expressed specific lytic function while in others did not. All of them did not expand during primary stimulation. An alternative possibility is that the E75 responders are not tolerized, but the intensity and duration of signaling by peptide–HLA-A2 complexes is insufficient to initiate T cell proliferation (18, 29, 30). This may indicate that the TCR interaction is of low affinity sufficient only to cause differentiation of responders in killer cells even in the absence of exogenous IL-2. As shown in **Fig. 5**, DC-NP/DC-E75±IL-12 did not increase the E75 specific $CD8^+$ CTL activity. This implies that IFN-γ secretion and cytokine–dependent proliferation of these cells require different levels of TCR engagement, which cannot be compensated by exogenous cytokine. This contrasts with the results reported for virally activated effector CTL *in vitro* and *in vivo* which show that similar levels of TCR engagement are required for IFN-γ secretion, cytotoxicity, and proliferation (3, 20, 24).

Thus the inability of E75 specific CTL to expand, may not be a consequence of low affinity TCR interaction. Importantly, anti B7.1 and anti B7.2 which stimulated the production of IFN-γ, did not affect the induction of specific cytotoxic activity of E75 stimulated $CD8^+$ cells, suggesting that IL-12 alone or together with B7.1 blocking are insufficient to completely reverse the state of partial T cell inactivation. Blocking of B7 was neither inhibitory for induction of CTL effector function as previously reported for naive, nor stimulatory as described for effector, $CD8^+$ cells in other systems (18, 31-34). It should be mentioned that residual NK cells as well as, T cells of other self-specificities may be responsible for a part of IFN-γ production in the absence of E75, and the increase in the non-specific CTL activity by addition of IL-12 and anti B7 (35). However, the levels of IFN-γ in response to E75+IL-12 + anti CTLA-4 suggest a synergistic rather than additive effect between E75 + anti CTLA-4 which is mediated only by T cells (35, 36).

The conclusion that IL-12 alone or together with IL-2 and cognate Ag cannot enhance cytotoxic function was strengthened by the fact that a slight increase in specific cytotoxic activity was observed with TNF-α in some of the donors tested (1 and 4). TNF-α did not synergized with IL-12 in induction of IFN-γ or enhancement of CTL activity suggesting that different signaling pathways activate IFN-γ secretion and cytotoxic function.

The identification of HER-2 specific $CD8^+$ effector cells in healthy donors, which, by functional criteria, define several subpopulations, is of interest for cancer vaccination and understanding immunity to human cancers. These populations of effectors may include few conventional memory cells (based on ^3H-TdR incorporation data) which require restimulation to divide and elicit effector functions (20). These populations include: *(a)* highly activated effectors such as the ones present in Donor 2, which secreted high amounts of IFN-γ and expressed specific CTL function even in the absence of IL-12 based on IFN-γ detection in 50 µl supernatant *(b)* partially activated effectors which express specific cytolitic function but required IL-12 for IFN-γ secretion (donors 1, 6); these effectors may resemble $CD8^+$ cells expressing "split anergy" *in vitro* (37); *(c)* less activated effectors which did not express specific cytolytic function. Some may secrete IFN-γ (Donor 3) but the majority require IL-12 for rapid IFN-γ secretion (Donors 4, 5, 6). A common characteristic of these populations is the poor IL-2 secretion and proliferating responsiveness to Ag + cytokines. By these functional criteria these populations may be partially similar with tolerized $CD4^+$ memory effectors (36, 38) because this form of anergy is not completely reverted by IL-12 and anti B7.1/CTLA-4, as described for the tolerized memory $CD4^+$ cells. It will be important to establish which of these subpopulations are endowed with protective function and how they can be expanded to mediate therapeutic effects.

Figure Legends

Figure 1. Freshly isolated plastic non-adherent PBMC specifically secrete IFN-γ in reponse to stimulation with E75. Potentiation by IL-12, anti B7.1 and B7.2 mAb. The HLA-A2 restricted HER-2 peptide E75, and the control peptides E39, D132 and F57 were used at a final concentration of 10 μM. IL-12 was used at 3 U (330 pg/ml). IL-2 was used in the experiments shown in parallel A and B at a final concentration of 5 Cetus units (30 IU/ml) for the last 8 h of culture before collection. Results in A, B and C are from separated experiments.

Figure 2. Early and rapid induction of IFN-γ in plastic non-adherent PBMC from Donor 3 is IL-12 and peptide concentration dependent. Results in panels A and B are from the same experiment. (A, B) All peptides were used at 10 μg/ml; IL-12 at 330 pg/ml (A-D). IL-12 and anti CTLA-4 mAb synergize in enhancing IFN-γ induction in response to E75 in peptide isolated $CD8^+$ $CD45RO^+$ cells. Equal numbers of $CD8^+$ $CD45RO^+$ and $CD8^+$ CD45RO cells were used as responders in each well. Experimental conditions were as described in the Materials and Methods.

Figure 3. Rapid IFN-γ secretion by E75+IL-2 stimulation in Donor 4 is mediated by $CD8^+$ cells. IL-12 synergize with anti B7.1/B7.2 in enhancing IFN-γ secretion. (A), (B) Isolated $CD8^+$ cells, (C) unseparated T cells. IL-12 was added to the DC together with the responders (s), 2 h before addition of responders (e), or 12 h after addition of responders (l). (B) Anti B7.1 and B7.2 were added to the DC 1 h

before addition of responders. ± cytokines indicates that in all cultures IL-2+TNF-α+IL-12 were either present or absent. (B) In the absence of cytokines E75 induced IFN-γ was detected in the presence of anti B7.1 at 48 h (50 pg/ml).

Figure 4. Stimulation with E75 plus IL-12 does not promote enhancement of Ag specific recognition. (A, B, C) primary stimulation of freshly isolated T cells with E75 pulsed on autologous DC generated by the $CD14^+$ method. Donor 1 (A), Donor 2 (B) unseparated T cells, (C) Donor 4 isolated $CD8^+$ cells. (A, C) E:T was 10:1, (B) E:T was 20:1. The values for the IFN-γ secreted in these experimental conditions are shown for Donor 2 in **Fig. 1C**, and for Donor 4 in **Figure 3A**. D132 and D125 are unnatural Muc-1 peptides used as specificity controls. Each experiment was repeated three times with similar results. One representative experiment is shown.

Figure 5. E75 stimulation in the presence of IL-12 and anti CTLA-4 or anti B7.1 enhanced only marginally $CD8^+$ cells proliferation. (A) Experimental conditions are described in **Figure. 2C** 48 h after stimulation, 10^5 Donors 3 live cells were incubated with of ^3H–TdR for 8 h. The experiment was preformed in tetraplicate wells. Differences in ^3H–TdR are significant for all the (±) E75 groups by the Student's t – test, but the stimulation indexes are ≤ 2.0. Equal numbers $CD45RO^+$ and CD45RO depleted (CD45RO) were collected from cultures after 48 h and used to determine differences in the rate of proliferation by E75 (B) Recognition of E75 was determined 48 h after stimulation. IL-2 and TNF-α were

not added to these cultures, E:T was 20:1 using equal numbers of recovered live cells as effectors.

Table I. Summary of the effector functions of CTL responding to E75 in different donors

Donors	- IL-12		+ IL-12+αB7.1/CTLA-4	
	IFN-γ	CTL	IFN-γ	CTL
1, 6	-	+	+ + +	+
2	+ +	+	+ + +	+
3	+	-	+ + +	-
4, 5, 7	-	-	+ + +	-
Patient 127	-	-	+ + +	-

a IFN-γ secretion was considered to be (+) if the cytokine levels in ELISA were ≥ 100 pg/ml/24 h from 1.75 x 10^6 cells/ml in the absence of exogenous IL-12.

b Specific CTL activity was considered to be (+) where specific recognition of E75 on T2 cells was ≥ 10% at an E:T ratio of 10:1 in the absence of E75 stimulation. Results are from Donors 1 and 6 (5 independent experiments), Donors 2, 3, 4, 5, 7 (3 independent experiments).

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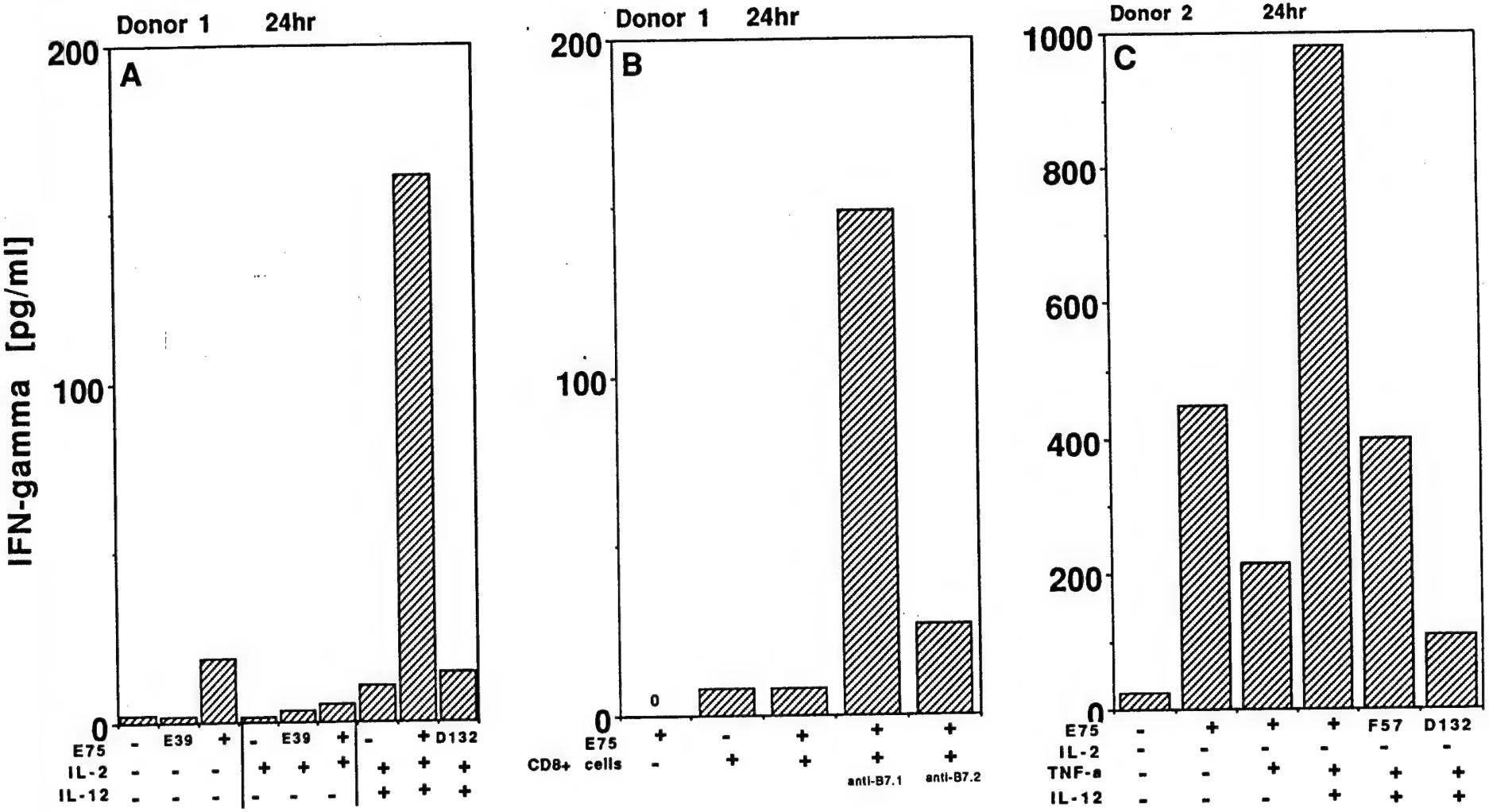
Figure 1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200
100
0
IFN-gamma [pg/ml]
E75 - E39 + - E39 + - + D132
IL-2 - - - + + + + + +
IL-12 - - - - - - + + +
Donor 1 24hr
B
200
100
0
0
E75 + - + + +
CD8+ cells - + + + +
anti-B7.1
anti-B7.2
Donor 2 24hr
C
1000
800
600
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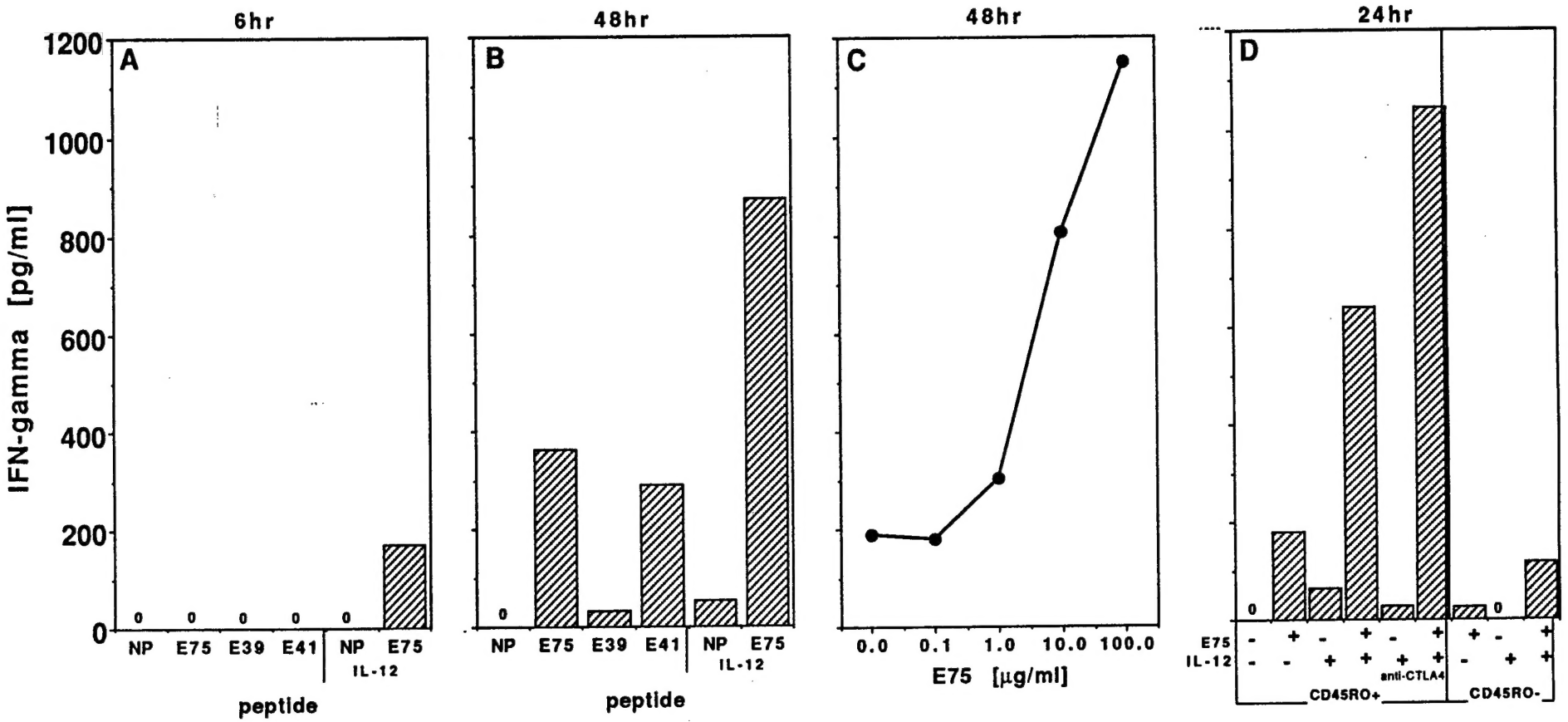
E75 - + + + F57 D132
IL-2 - - - - - -
TNF-a - - + + + +
IL-12 - - - + + +

Figure 2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48hr
48hr
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A
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D
IFN-gamma [pg/ml]
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1000
800
600
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NP E75 E39 E41 NP E75
IL-12
peptide
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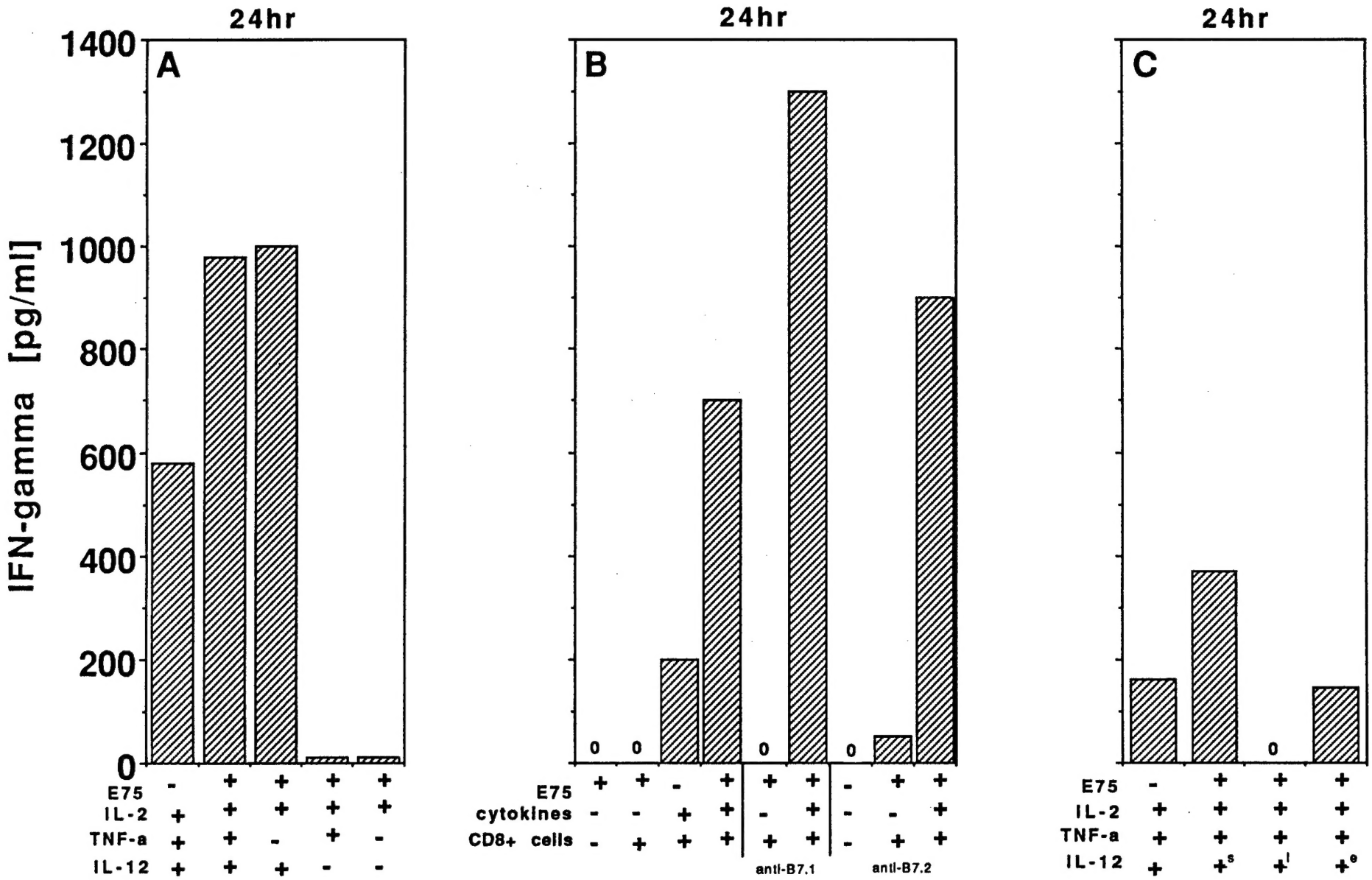
IL-12
anti-CTLA4
CD45RO+
CD45RO-

Figure 3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IFN-gamma [pg/ml]
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cytokines
CD8+ cells
anti-B7.1
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Figure 4

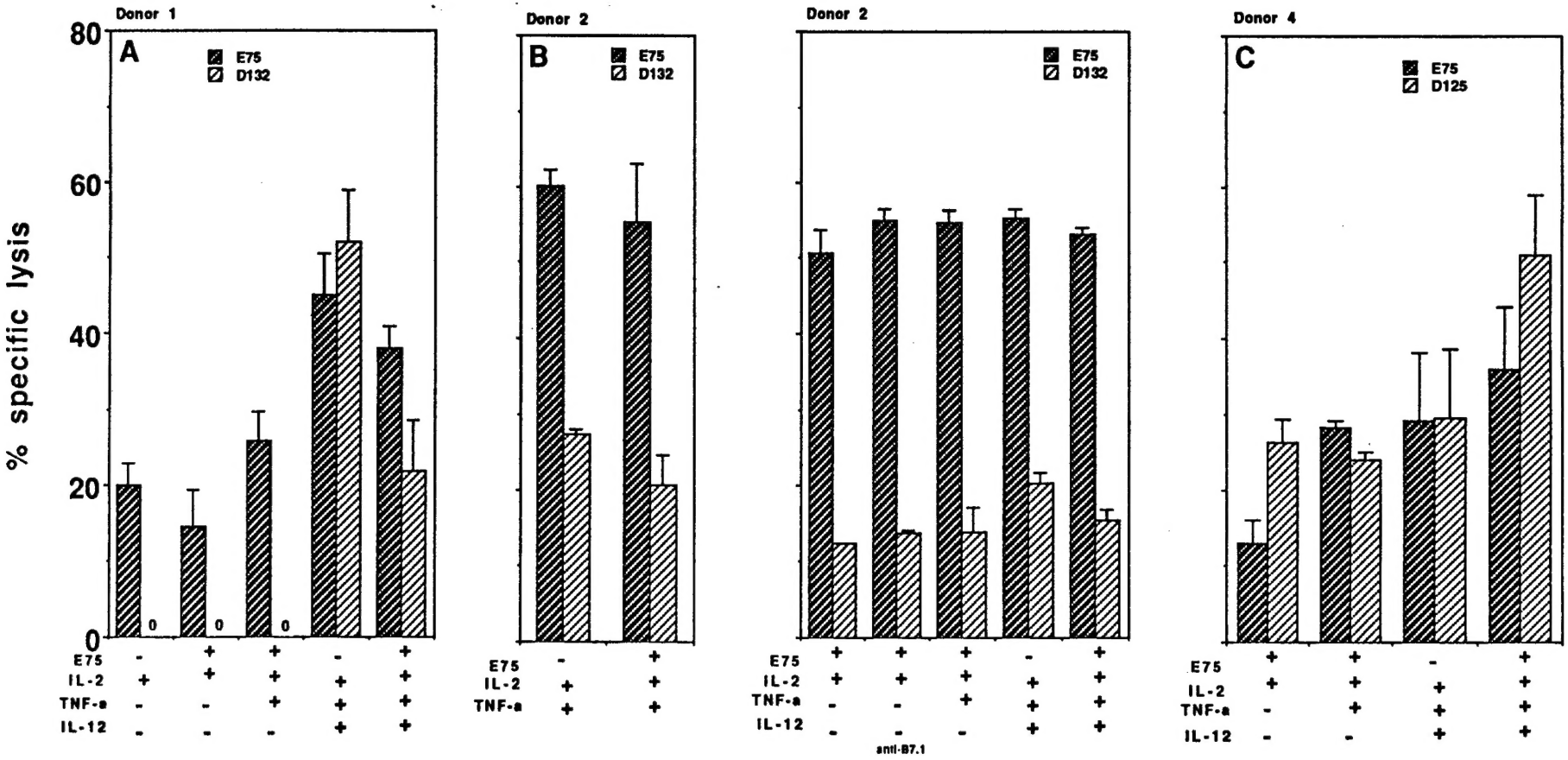
% specific lysis
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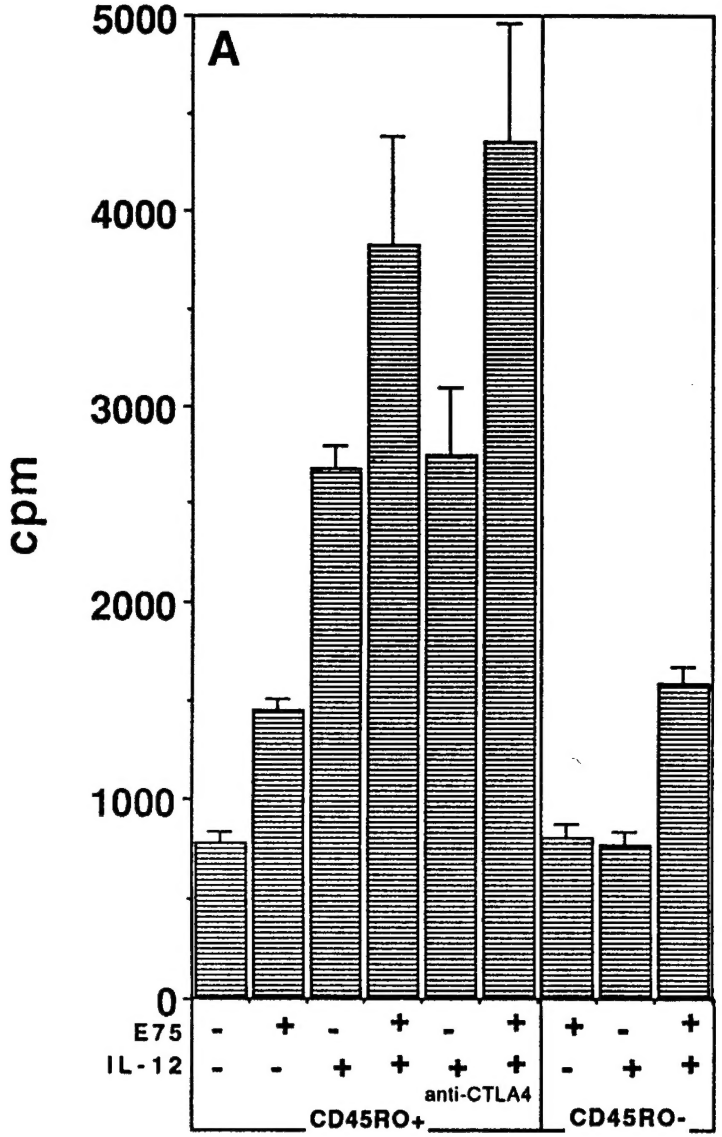
Figure 5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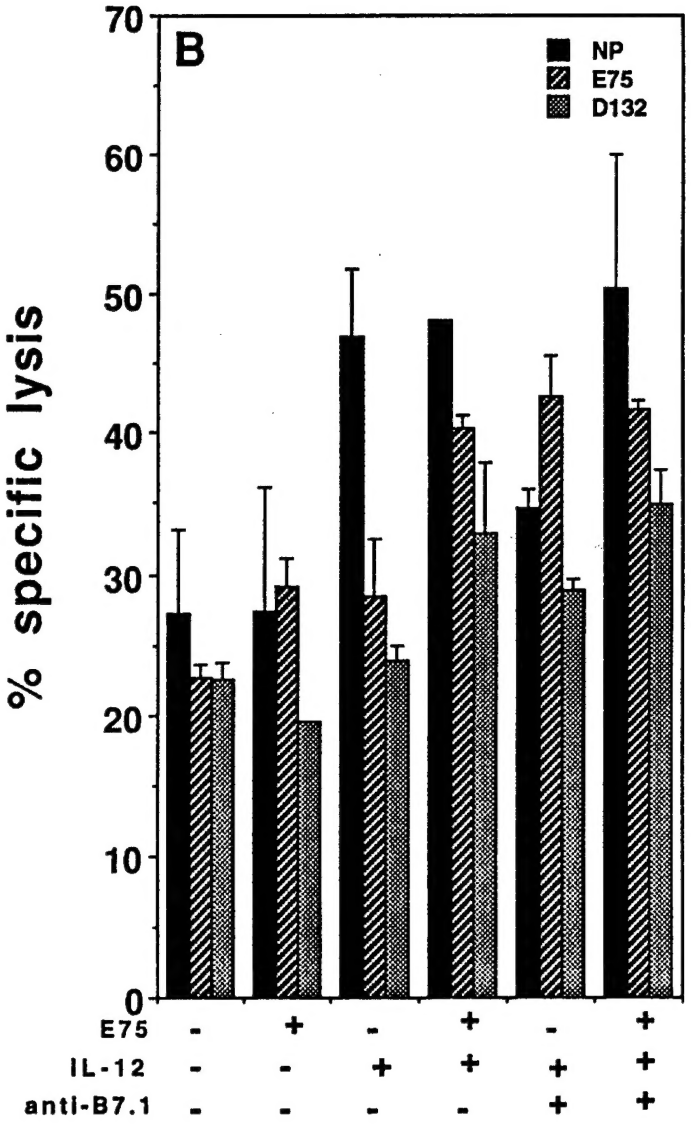
B
NP
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D132
% specific lysis
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